Júlio Henrique Livramento dos Santos

Biopatologia do cancro da bexiga associado ao *Schistosoma haematobium* : efeito cancerígeno possível mediado por aductos de estrogénio. Um ensaio clínico numa área endémica de schistosomose urinária em Angola

Tese de Candidatura ao grau de Doutor em Ciências Médicas submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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(William Osler. Aequanimitas; The master world in medicine)

Para a minha mãe

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Gouveia MJ, **Santos J**, Brindley PJ, Rinaldi G, Lopes C, Santos LL, da Costa JM,Vale N. Estrogen-like metabolites and DNA-adducts in urogenital schistosomiasis-associated bladder cancer. *Cancer Lett*. 2015 Apr 10; 359(2):226-32.

Resumo

A schistosomose, no sentido clínico lato do termo, é uma doença tropical negligenciada e é considerada a doença humana por helmintos mais importante em termos de morbidade e mortalidade. Estratégias de controlo diversas têm sido desenvolvidas no sentido de bloquear a transmissão e reduzir a carga parasitária dessa doença. No entanto, a schistosomose, particularmente aquela que nos ocupa, a schistosomose urogenital (UGS), continua a ser um problema de saúde pública, especialmente nas regiões rurais da África subsariana, e, particularmente em Angola. Além disso, o cancro de bexiga é uma complicação frequente e grave da UGS. Com efeito, a Agência Internacional de Investigação do Cancro da Organização Mundial de Saúde (IARC/OMS) classifica a infeção por *Schistosoma haematobium* como causa definitiva de cancro, i.e., carcinogéneo do grupo 1. A gravidade e a frequência das sequelas da UGS e suas complicações, incluindo o carcinoma espinocelular ou epidermoide da bexiga estão relacionados com a carga e a duração da infeção. Metabólitos de estrogénio, nomeadamente na sua forma molecular como catecol e de aductos de ADN, estão implicados, pelo menos em parte, nos mecanismos da carcinogénese induzida pela UGS. Os resultados de análises por LC-MS/MS realizadas em amostras de urina de 40 pacientes angolanos diagnosticados com UGS, metade dos quais apresentando UGS associada a carcinoma espinocelular ou carcinoma urotelial, permitiram verificar que os cromatogramas obtidos revelaram: i) a presença de inúmeros metabólitos de estrogénio, incluindo sete especificamente identificada nos casos de UGS, por comparação com um banco de dados de metabólitos na urina de seres humanos saudáveis; ii) inclusão de moléculas de catecol estrogénio e aductos de ADN, dois dos quais tinham sido identificadas anteriormente em vermes adultos e ovos de *S. haematobium*; iii) a presença associada da molécula 8-oxodG, notável indicador de lesão do ADN; iv) a evidência de diferenças substanciais no perfil cromatográfico dos metabólitos entre o grupo de doentes UGS com cancro e sem cancro. Paralelamente, em biópsia da mucosa vesical destes pacientes, a p53 foi profusamente detetada, por imunohistoquímica, e, numa parte muito significativa destes doentes a p53 está alterada. Os resultados obtidos parecem suportar o postulado que atribui a metabólitos de estrogénio um papel relevante na iniciação da carcinogénese vesical associada à UGS e, estudos posteriores sobre o comportamento e metabolização destes compostos pode perspetivar melhorias na compreensão dos mecanismos moleculares envolvidos na carcinogénese e como biomarcadores para diagnóstico e/ou prognóstico de cancro.

Abstract

Schistosomiasis is a neglected tropical disease and is considered the most important human disease caused by Helminths in terms of morbidity and mortality. Several control strategies have been developed in order to block the transmission and reduce the burden of this disease. However, schistosomiasis, particularly the one that occupies us, the urogenital schistosomiasis (UGS), remains a public health problem, especially in rural areas of sub-Saharan Africa, particularly in Angola. In addition, bladder cancer is a frequent and serious complication of UGS. Indeed, the International Agency for research on cancer of the World Health Organization (IARC/WHO) classifies the infection by *Schistosoma haematobium* as a cause of cancer, i.e., group 1 carcinogen. The severity and frequency of *sequelae* of UGS and its complications, including squamous cell carcinoma (SCC) of the bladder are related to the load and the duration of the infection. Oestrogen metabolites, in particular molecular form as catechol and DNA adducts, are involved, at least in part, on the mechanisms of carcinogenesis induced by UGS. The results of analysis by LC-MS/MS carried out in urine samples of 40 Angolan patients diagnosed with UGS, half of which featured UGS-associated with SCC or urothelial carcinoma, enabled verify that the chromatograms obtained revealed: i) the presence of numerous oestrogen metabolites, including seven specifically identified in cases of UGS, by comparison with a database of metabolites in the urine of healthy human beings; ii) inclusion of catechol oestrogen molecules and DNA adducts, two of whom had been identified previously in adult worms and eggs of *S. haematobium*; iii) the presence of the molecule 8-oxodG, noteworthy DNA injury indicator; iv) substantial differences in chromatographic profile of metabolites between the groups of UGS patients with cancer and without cancer. At the same time, in bladder mucosal biopsy of these patients, the p53 was profusely detected by immunohistochemistry, and, in a significant part of these patients, p53 is altered. The results seem to support the premise that assigns the oestrogen metabolites played an important role in the initiation of bladder carcinogenesis associated with UGS, and subsequent studies on the behaviour and metabolism of these compounds can drive improvements in order to a better understanding of the molecular mechanisms involved in carcinogenesis induced by UGS and as biomarkers for diagnosis and/or prognosis of cancer.

LISTA DE ABREVIATURAS

ADN/DNA – Ácido Desoxirribonucleico
Bcl-2 - B-cell lymphoma 2
CE – Exame por cistoscopia
CHO – Chinese hamster ovary cells
CHR – Reação da Tumefação das cercarias (Cercarien Hullen Reaktion)
CIBP – Centro de Imunologia e Biologia Parasitária, INSA,I.P.
COMT - Catecol-O-metiltransferase
CYP19 – Aromatase de Coenzima P450
CYP1A – Oxidase de Coenzima P450
CYP3A - Oxidase de Coenzima P450
DALYs - disability-adjusted life years
DTS-Doença de Transmissão Sexual
E2 – estradiol
E1 - Estrona
ELISA – Ensaio Imunoenzimático (Enzyme Linked Immunosorbent Assay)
ES – excretion/secretion products
FGS – Schistosomose Genital Feminina (Female Genital Schistosomiasis)
GST - glutatião-s-transferase
HAB – Hospital Américo Boavida
HIV/AIDS – Human immunodeficiency virus / Acquired immune deficiency syndrome
HPLC - High-performance liquid chromatography
HPV – Human Papiloma Virus
IARC – International agency for research on cancer
KRAS - Kirsten rat sarcoma viral oncogene homolog
LC-ESI-MS - Liquid chromatography−electrospray ionization−mass spectrometry
LC-MS/MS - Liquid chromatography-mass spectrometry
LH - Luteinizing Hormone
MINSA – Ministério da Saúde de Angola
NIH – National Institute of Health, USA.
2-OHE1 (E2) e 4-OHE1 (E2) - catecol estrogénios,
OMS – Organização Mundial da Saúde
p27- proteína inibidora de quinase 1 (Kip1)
p53 – proteína supressora de tumores
PCR – Reação em cadeia da Polimerase (Polymerase chain reaction)
PZQ – Praziquantel

SCC – Carcinoma de células escamosas (Squamous cell carcinoma)

SEA – Soluble Eggs Antigens

SIDA – Síndrome de Imunodeficiência Adquirida

S-phase – synthesis phase

SWAP – Schistosome worm antigen preparation

TCC – Carcinoma de células trasicional (Trasitional cell carcinoma)

TP53 – gene que codifica a p53

UE – Urografia de eliminação

UGS – Schistosomose urogenital (urogenital schistosomiasis)

UNDP – United Nations Development Programme

US- Ultrassonografia

USA – United States of America

UV – Ultraviolet

VIH – Vírus de Imunodeficiência Humana

WHO – World Health Organization (Organização Mundial de Saúde)

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Capítulo 1

Introdução e Generalidades

1.1. A SCHISTOSOMOSE UROGENITAL

A Schistosomose Urogenital (UGS no acrónimo inglês; *urogenital schistosomiasis*) é a entidade nosológica subsequente à infeção por *Schistosoma haematobium*. É consensual, esta infeção parasitária é exclusivamente humana. Muitos casos de infeção por *S. haematobium* cursam com sintomas mínimos. No entanto, uma parte significativa dos pacientes, estimada entre 25% a 50%, pode desenvolver infeção com morbilidade moderada a grave (CH King, 2010). Manifestações crónicas da doença incluem disfunção renal, obstrução pieloureteral e carcinoma de células escamosas da bexiga. Tal facto deriva, julga-se, do depósito de ovos na mucosa da bexiga e do trato urinário e do processo inflamatório crónico a que ele dá origem. (Hodder et al., 2000; Parkin, 2002). Mas a abrangência clínica da infeção pode ser maior: 75% das mulheres infetadas sofrem de schistosomose genital feminina (FGS no acrónimo inglês) do trato genital inferior (Hotez et al., 2009). Também aqui, a FGS resulta do depósito de ovos do parasita no útero, colo do útero, vagina e vulva, com resposta inflamatória subsequente, consequências na fertilidade (Santos et al., 2014) e maior susceptibilidade da mulher à infeção pelo VIH (Kjetland et al., 2008; Ndhlovu et al., 2007; Jourdan, 2012).

O carcinoma espinocelular da bexiga é uma complicação frequente e grave associada à UGS. A descrição de casos clínicos na literatura sugere que pacientes com UGS podem desenvolver o carcinoma mais cedo do que as pessoas não infetadas. A gravidade e a frequência das sequelas da UGS e de suas complicações estão relacionados com a intensidade e a duração da infeção (Porta et al., 2011). Acresce o facto de o *S. haematobium* ser classificado como carcinogéneo do grupo 1 pela Agência Internacional para a Investigação do Cancro (IARC/OMS, 2012), embora os mecanismos celulares e moleculares envolvidos não estejam completamente definidos (Botelho et al., 2009). Recentemente foi postulado, pelo grupo de investigação onde me integro, o papel relevante, em parte, de metabólitos de estrogénio como iniciadores do carcinogénese vesical associada à infeção (Correia da Costa et al., 2014). A presente tese consubstancia, entre outros, um exercício de aductómica – neologismo emergente para designar o estudo de metabólitos de estrogénio, e de outros compostos químicos indicadores de dano genético no hospedeiro e excretados na urina - num grupo bem caracterizado de doentes com UGS de Angola. Nos capítulos subsequentes desta tese reportamos os nossos resultados, antecipando duas questões transversais a este exercício: i) suportam eles tal postulado? E, de um ponto de vista clínico, podem ser úteis na previsão da evolução para cancro, como biomarcadores? Antecipemos, agora, alguma informação da literatura sobre os parasitas antropófilos do género *Schistosoma* e sobre a Schistosomose no seu sentido clínico lato.

1.1.1. Sistemática e Taxonomia do Gênero *Schistosoma*.

Schistosomose ou Bilharziose designa o complexo de infeções parasitárias, na sua forma clínica aguda e crónica causada por vermes digenéticos do género *Schistosoma*. Posição sistemática: **Reino:** ANIMALIA; **Filo:** PLATHYHELMINTHES; **Superclasse:** EUPLATHYHELMINTHES; **Classe:** TREMATODA; **Subclasse:** DIGENEA; **Ordem:** STRIGEIFORMES; **Família:** *Schistosomatidae*; **Subfamília:** *Schistosomatinae*; **Género:** *Schistosoma* **Espécie:** *Schistosoma haematobium*, Bilharz (1851); *Schistosoma mansoni*, Sambon (1907); *Schistosoma japonicum*, Katsurada (1904); *Schistosoma intercalatum*, Fisher (1934); *Schistosoma mekongi*, Voge, Bruckner & Bruce (1978). Os membros da família *Schistosomatidae* apresentam sexos separados, são dioicos. Distinguem-se três subfamilias, *Shistosomatinae*, *Bilharziellinae* e *Gigantobilharzinae* (Cook & Zumla, 2009). Dos 12 géneros da subfamília *Schistosomatinae*, vários estão confinados a aves e cinco a mamíferos. Só 5 espécies de *Schistosoma* estão associadas à infeção humana (Cook & Zumla, 2009). *Schistosoma malayensis*, como nova espécie, aguarda consenso.

1.1.2. Nota histórica

Investigações arqueológicas e antropológicas recentes sugerem o conhecimento dos sintomas da Schistosomose, como entidade nosológica distinta, na África do Norte, na alta antiguidade, e na China. Contudo, não existem referências relativas à sua ocorrência noutras regiões de África.

Os Papiros do Antigo Egipto. As referências à doença datam do período Arcaico. Através de estudos paleográficos e paleo-epidemiológicos, numerosas informações testemunham esse conhecimento, nomeadamente, pela demonstração da presença de ovos de *Schistosoma haematobium* calcificados nos rins (Ruffler, 1910) e de antigénios circulantes em múmias da 20ª dinastia (Colley,1996). Outros autores interpretaram os caracteres hieroglíficos mencionados em antigos papiros (Ebers, Hearts e Kahum), como sendo uma referência à hematúria causada pela infeção por *S. haematobium*, ou sequer, o uso de compostos antimoniais no tratamento da hematúria. Sabendo que até há cerca de 35 anos, os compostos antimoniais eram os fármacos de eleição na quimioterapia da schistosomose devido à sua elevada ação parasiticida, esses escritos constituem-se como provas indeléveis que confirmam os conhecimentos existentes nesse período sobre a doença e sua importância (Cook & Zumla, 2009).

O parasita nos nossos dias. Theodore Bilharz, 1851, patologista alemão, no decurso de exames *pós-morte* efetuados em soldados egípcios, no Cairo, descreveu, em carta a seu mestre na Alemanha, o parasita putativamente responsável pelas lesões posteriormente referidas como a schistosomose urogenital. Designou-o como *Distomum.* Mais tarde,

constatadas diferenças substanciais e distintas do novel parasita relativamente às espécies do género *Distomum*, Weinland, 1858, propôs um novo género: *Schistosoma* (do grego *schistos*=fenda e *soma*=corpo); em homenagem a Theodore Bilharz, Cobbold em 1859, propôs a alteração do género *Schistosoma* para *Bilharzia*. Apesar da frequente referencia à doença de bilharziose, na literatura Francesa e Portuguesa, prevalece a designação *Schistosoma*. Harley e Cobbold em 1859 indicam que a infeção humana ocorria por via cutânea; Manson, 1902, sugeriu a existência de duas espécies distintas de *Schistosoma*; posteriormente, foi proposta a designação de *S. mansoni* para a espécie com ovos de esporão lateral; Leiper,1915, estabeleceu a existência de duas espécies distintas do género *Schistosoma* e referiu que, no ciclo de vida do parasita, havia um hospedeiro intermediário - um molusco de água doce; Fisher, 1934, identificou uma nova espécie, com os ovos de esporão terminal e eliminados com as fezes, propondo o nome de *S. intercalatum*; a ocorrência de schistosomose no delta do rio Mekong data de 1957; Barbier, 1966, identificou um foco no Sul de Laos; Voge, Bruckner e Bruce, 1978, descreveram o agente causal como uma nova espécie: *S. mekongi*. (Colley, 1996, 2014; Cook & Zumla, 2009)

A Schistosomose na Ásia. Admite-se também o conhecimento da schistosomose na China na antiguidade (Mao & Shao, 1982). O prurido de Kabure ou a síndrome de Katayama, foram descritas pela primeira vez em 1847, numa aldeia do distrito de Hiroshima, no Japão; a espécie *S. japonicum* só foi descrita em 1904, na sequência das observações de Katsurada; posteriormente, Fujinamie e Nakamura, 1909, estudaram a via de transmissão desta espécie ao hospedeiro vertebrado, expondo cães e gatos nos campos de arroz onde ocorriam os casos humanos de schistosomose. Após essa exposição observaram ovos de *S. japonicum* nas fezes daqueles animais e, 40 dias após a infeção os animais foram sacrificados e autopsiados, sendo os vermes recolhidos das veias do sistema porta, o que confirmou a existência do parasita no vertebrado. O *Schistosoma japonicum* é a única espécie zoonótica do género *Schistosoma*. (Cook & Zumla, 2009).

A schistosomose tem significado histórico-militar. Durante a invasão Napoleónica do Egipto entre 1799-1801, as tropas francesas infetaram-se por *S. haematobium*. No corpo médico militar francês, uns atribuíram a hematúria à transpiração e ao clima do Egipto, outros à “vingança dos Faraós”. Durante a Segunda Guerra Mundial, mais de 1300 soldados americanos infetaram-se com *S. japonicum* durante a invasão de Leyete nas Filipinas. Alguns anos mais tarde, durante os treinos militares preparatórios para a invasão de Taiwan, executados na bacia do Rio Yangtze, soldados do Exército Vermelho da República Popular da China ficaram expostos à infeção por *S. japonicum*. Muitos

soldados desenvolveram a Febre de Katayama, ocorrência que motivou o adiamento do assalto anfíbio planeado à ilha (Vieira, JM Costa & JH Cross, 2006).

1.1.3. Aspetos de Epidemiologia

A schistosomose é a segunda infeção parasitária, depois da Malária, com grande significado em termos de morbilidade e mortalidade, e de impacto em saúde pública. Admitem-se cinco espécies do género *Schistosoma* na sua etiologia: *S. haematobium, S. mansoni, S. japonicum, S. intercalatum* e *S. mekongi*. De acordo com estimativas da OMS de 2006, a schistosomose afeta 200 milhões de pessoas em todo o mundo, das quais 20 milhões apresentam formas clínicas graves (Figura 1). Em termos de mortalidade, é estimado o valor de 500.000 casos anuais, e a população em risco de infeção rondará os 600 milhões. Hotez & Fenwick, 2009, estimam que 93% das pessoas infetadas vivem no continente africano, cerca de 192 milhões. Em 2012 a OMS estimava em, apenas 15% o número de pacientes com acesso a tratamento. O Banco Mundial, 1995, estimou a população residente nos países endémicos num valor de 3.7 biliões de pessoas. Este número tem crescido enormemente nos últimos anos, de acordo com estimativas de 1997 da mesma instituição Internacional. Numa revisão recente de King *et al*. (2008) é claramente demonstrado que o número de DALYs (*disability adjusted life years*) causado pela infeção está subestimado. Este indicador é utilizado para quantificar o impacto das doenças na vida do indivíduo, ou seja, o número de anos de vida produtiva que uma pessoa perde devido à doença, o que reforça o seu impacte na população; foi estimada em 70 milhões de DALYs perdidos anualmente só em África. Se confirmadas, as previsões de Charles King, a UGS pode representar a infeção mais comum na África Subsariana.

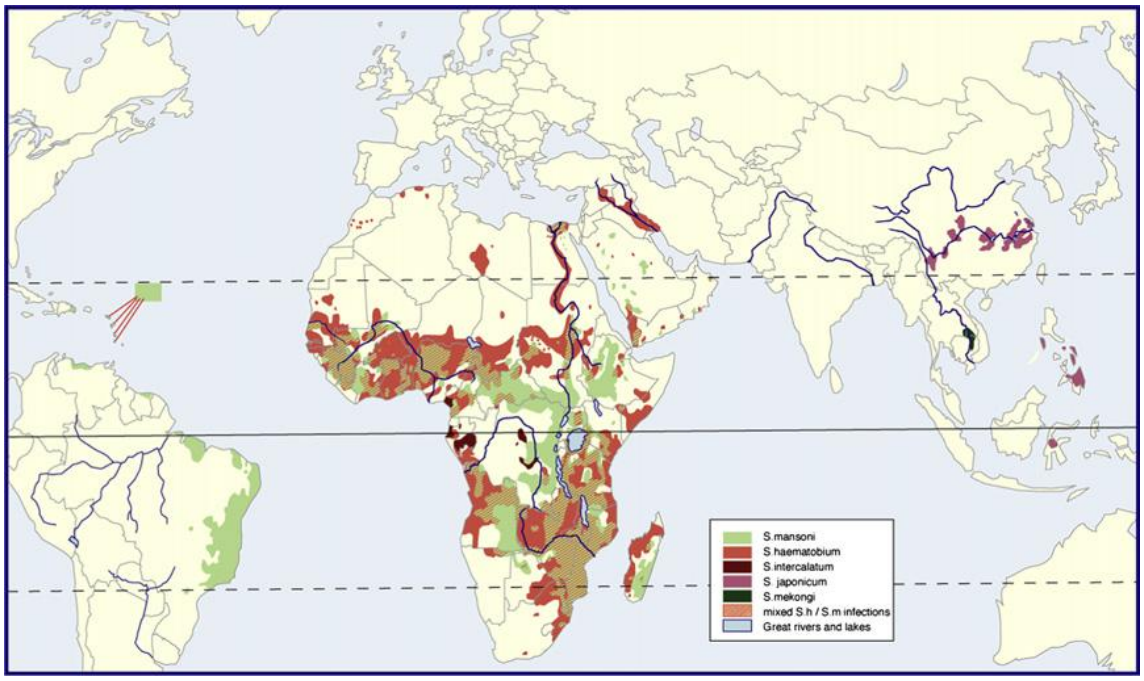

Figura 1. Distribuição global da Schistosomose.
(Com a permissão da Elsevier e a cortesia do Professor B. Gryseels).

1.1.4 Aspetos de biologia e ciclo de vida

Os vermes adultos do gênero *Schistosoma* vivem imersos no sangue no lúmen de vasos sanguíneos. De modo diferente de outros Platelmintes, estes parasitas são digenéticos, planos e alongados; assumem, no entanto, uma forma arredondada como forma de adaptação à permanência nos vasos sanguíneos do trato geniturinário ou gastrointestinal. Apresentam um ciclo de vida heteroxénico, implicando um molusco invertebrado como hospedeiro secundário: caracóis de água doce do género *Bulinus*, *Oncomelania*, ou *Biomphalaria*, de acordo com a espécie de *Schistosoma*. Assim, a sua distribuição geográfica e a manutenção da infeção humana é dependente e é limitada pela presença de um hospedeiro secundário susceptível.

Vermes adultos de *Schistosoma* são sexualmente distintos, (uma característica que os separa de outros Platelmintes, habitualmente hermafroditas). O macho apresenta um sulco ventral desde a ventosa ventral até à extremidade posterior, formando o canal ginecofórico, também designado de bolsa copuladora, onde a fêmea é mantida durante e após a cópula. A biologia do género *Schistosoma* difere de outros Platelmintes também no modo de infeção do hospedeiro definitivo: a penetração de cercarias faz-se através da pele (*per cutem*) em vez da ingestão oral (*per os*). As espécies de *Schistosoma* antropófilas podem infetar outros vertebrados, constituindo-se estes animais como reservatório biológico da infeção; estes factos têm importância epidemiológica somente para *S. japonicum* e, possivelmente, *S. mekongi*. Caracóis do gênero *Biomphalaria*,

Bulinus, *Neotricula* e *Oncomelania* são os principais hospedeiros intermediários respetivamente para *S. mansoni*, *S. haematobium*, *S. mekongi* e *S. japonicum* (Butterworth et al., 1988).

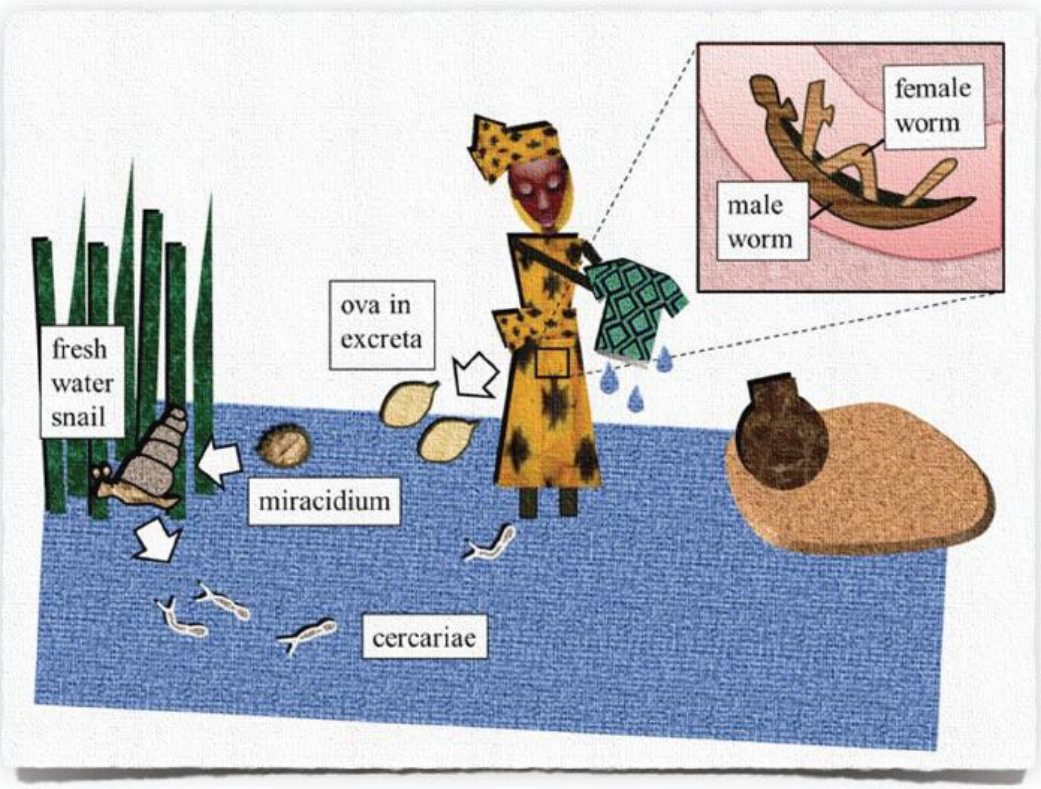

Figura 2. Ciclo de vida do *Schistosoma haematobium*.
(Com a permissão e a cortesia do Dr. Peter Mark Jourdan)

O ciclo de vida do *Schistosoma* spp. começa assim que as cercarias, forma larvar livre, entram no corpo humano após penetração através da pele (Figura 2). As cercarias são constituídas por uma cabeça e uma cauda bifurcada (furo cercarias). Em contacto com a pele humana, atraídas pelo calor corporal (37ºC), perdem a cauda e só a cabeça continua a migração tecidual até encontrar um vaso sanguíneo. São agora designadas *schistosomulae*, que migram para os pulmões e depois para o fígado, onde amadurecem, tornando-se em vermes adultos em 6 semanas. Posteriormente o macho toma a sua fêmea e desce para posições terminais na circulação venosa (Figura 3).

Figura 3 – Vermes adultos.

Vermes adultos de *S. haematobium* são encontradas principalmente nos plexos venosos da bexiga, próstata e útero, enquanto o *S. japonicum* e *S. mansoni* são observados na veia porta e veias mesentéricas (Butterworth et al., 1988; Ouma et al., 2001). Não são conhecidas as razões pelas quais as espécies selecionam estes habitat. Os vermes adultos podem viver nestas condições por mais de 30 anos (Vieira et al., 2007) com fêmeas libertando ovos entre 300-3000 por dia. Os ovos atravessam a parede da vénula para entrar no estroma. Estes segregam substâncias que promovem a desintegração dos tecidos, permitindo-lhes atravessar o estroma e alcançar o lúmen do órgão em que eles são depositados: o lúmen da bexiga urinária (*S. haematobium*) ou do cólon (*S. mansoni* e *S. japonicum*). Aqui amadurecem originando, em cerca de 10 dias, um *miracidium*. Os ovos são excretados pelas fezes (*S. mansoni*, *S. intercalatum*, *S, japonicum*) ou pela urina (*S. haematobium*) (Figura 4).

Figura 4. Ovos de *S. haematobium*. A, ovo fértil com *miracidium*; B, ovo calcificado. 100x (Original)

No exterior e em contacto com a água doce, o *miracidium*, forma larvar ciliada, sai do ovo e move-se ativamente em busca do molusco hospedeiro susceptível. Entra nele pelo pedúnculo, e aí se desenvolvem várias gerações de esporocistos (mães e filhas), por divisão assexuada, e, posteriormente em cercarias, em 4-6 semanas. As cercarias deixam o caracol e partem em busca de novos hospedeiros definitivos para reproduzir e manter a espécie (Butterworth et al., 1988; Ouma et al., 2001). No entanto, quase 50% dos ovos eliminados pelas fêmeas são retidos no estroma tecidual, constituindo a principal causa de patologia na Schistosomose.

1.1.5 A Schistosomose Urogenital: clínica e patologia.

A Organização Mundial de Saúde recomenda, desde 2009, que a infeção causada pelo *S. haematobium* se designe Schistosomose Urogenital, substituindo designações como Schistosomose Urinária ou Schistosomose Vesical (OMS, 2009). A doença de etiologia *S. mansoni* ou *S. japonicum* é designada de Schistosomose Intestinal. A presente tese tem como domínio fundamental, previamente o referimos, a Schistosomose Urogenital. A infeção pelo *S. haematobium* pode apresentar-se com sintomas mínimos, hematúria, disúria, anemia e inflamação do trato urinário. No entanto, parte significativa dos pacientes evolui para situações clínicas crónicas com lesões inflamatórias e calcificações no trato urinário, e subsequente uropatia obstrutiva e hidronefrose, lesões renais, como pielonefrite aguda e crônica e, uma predisposição para carcinoma das células escamosas da bexiga urinária (King, 2006). Uma revisão restrita de estudos de autópsias humanas sugere que as lesões na bexiga induzidas pelo *S. haematobium* ocorreriam em três fases: pólipos, manchas de fibrose e finalmente "*sandy patches*", manchas de fibrose granulares (Smith, 1975, Cheever, 1978). Finalmente, algum consenso subsiste: a evolução da patologia na Schistosomose Urogenital (UGS) depende da localização focal e da intensidade da infeção (Cheever, 1974, 1977, 1978).

1.1.6 A Histopatologia da Schistosomose Urogenital

A doença subsequente à infeção por *S. haematobium* é primariamente causada por uma reação de hipersensibilidade retardada (tipo IV) ao depósito de ovos na mucosa da bexiga e posterior formação do granuloma periovular. A longa permanência dos vermes e o depósito de ovos são favorecidas pela evidência de mecanismos de evasão imunitária, nomeadamente os designados por mimetismo molecular (Capron, 1998). O granuloma periovular é o achado histopatológico dominante na Schistosomose Urogenital; este granuloma é consequência da resposta imune dos hospedeiros, mediada por linfócitos T, contra produtos excretados pelo ovo e produzidos pelo *miracidium;* consiste predominantemente de células mononucleares, ou seja, de linfócitos e células

plasmáticas, e monócitos, macrófagos e fibroblastos, e alguns neutrófilos, eosinófilos e mastócitos (Kassis, 1978).

1.1.7 A Schistosomose genital feminina

A schistosomose genital feminina (SGF) é um achado frequentemente encontrado em mulheres infetadas por *S. haematobium*. O depósito dos ovos nestes tecidos, e subsequente formação de granulomas periovulares, origina lesões genitais referidas na literatura como salpingite, endometrite, vulvovaginite e cervicite. No homem são também frequentes achados similares caracterizados como epididimite, vesiculite e prostatite. Podem, estas lesões em ambos casos, causar infertilidade secundária, (Rey, 2001 e Bichler et al., 2006) e, favorecer expansão de doenças de transmissão sexual (DTS), especificamente o vírus de imunodeficiência humana (VIH), acelerando a progressão para a doença, bem como o papiloma vírus humano (HPV) (Leutscher et al., 1997; Ndhlovu et al., 2007 e Smith et al., 2008). Em zonas endémicas de UGS do continente africano, foram identificadas alterações histopatológicas causadas por *S. haematobium* compatíveis com FGS, em exames *post-mortem* realizados em mulheres em idade reprodutiva (Poggensee et al., 2001). A hemoespermia pode ser observada na fase inicial da infeção (Leutscher et al., 1998, 2005).

1.1.8 O Diagnóstico laboratorial da Schistosomose Urogenital

A observação microscópica de ovos de *S. haematobium* em sedimentos ou filtrados de amostras de urina, ou em tecidos colhidos após biópsia e convenientemente corados, constitui-se como o elemento de diagnóstico laboratorial definitivo. A OMS recomenda que o exame parasitológico para a deteção de ovos de *S. haematobium* seja executado a partir da filtração de 10 mL de urina dos pacientes utilizando para o efeito um filtro de policarbonato com tamanho de poro de 12 µm de diâmetro (Nucleopore). Após conveniente eluição do filtrado, as lâminas são observadas ao microscópio com ampliação de 100 x (OMS, 2009). Este método permite quantificar a carga parasitária; as infeções poderão ser categorizadas como leves em doentes que eliminam diariamente 1 a 10 ovos/10 ml de urina, como moderadas nos que eliminam de 11 a 50 ovos/10 ml e intensas quando superiores a 50 ovos/10 ml de urina (Rollinson *et al.,* 2005, Sousa Figueiredo *et al.*, 2009). A pesquisa de anticorpos específicos ou de antigénios circulantes pode ser executada pela utilização de diversos tipos de testes serológicos: *Western blott*, *Enzyme-Linked Immunosorbent Assay* (ELISA), Imunofluorescência (IF), *Radioimunoassays* (RIA) e *Cercarien Hullen Reaction* (CHR); ou ainda a pesquisa de sequencias especificas de ADN parasitário em sangue ou urina, utilizando diversos protocolos de PCR (*polymerase chain reaction*) (Felleisen R et al., 1992; Cook & Zumla,

2009). Algumas vantagens e muitas imperfeições atribuídas a cada um destes testes, *per si*, acentuam uma profunda falta de consenso. A pesquisa de hematúria e albuminúria, muito utilizada em estudos epidemiológicos nas áreas endémicas, sobretudo na população infantil, podem ser indicadores complementares de alguma utilidade; o mesmo para a identificação de eventual eosinofilia. Retenhamos, no entanto, que mau grado algumas imperfeições evidentes, os exames diretos para pesquisa de ovos são a primeira escolha para diagnóstico laboratorial.

1.1.9 O Diagnóstico imagiológico e endoscópico

O diagnóstico imagiológico é um precioso elemento complementar para a monitorização clinica da UGS particularmente nos aspetos da patologia dos tratos urinário e genital, e na avaliação após a terapia. Adiante, daremos conta da nossa experiência e dos resultados obtidos com a utilização da Ultrassonografia (US) e cistoscopia na monitorização clínica dos nossos pacientes com UGS (Capítulo 3). Vejamos, no entanto, algumas referências da literatura. A radiografia do trato urinário simples e contrastada pode ser um meio de diagnóstico importante na avaliação de sequelas e complicações da UGS; uma boa execução e observação experimentada permitem verificar, por exemplo, a calcificação linear da bexiga e dos ureteres. Aqui, a apresentação clássica de uma bexiga calcificada assemelha-se a uma cabeça fetal na pélvis. Muitos autores aceitam esta evidência como o sinal patognomónico de UGS, conhecido como “bexiga de porcelana” (Figura 5).

Figura 5. Rx simples do trato urinário : “bexiga de porcelana” (Original)

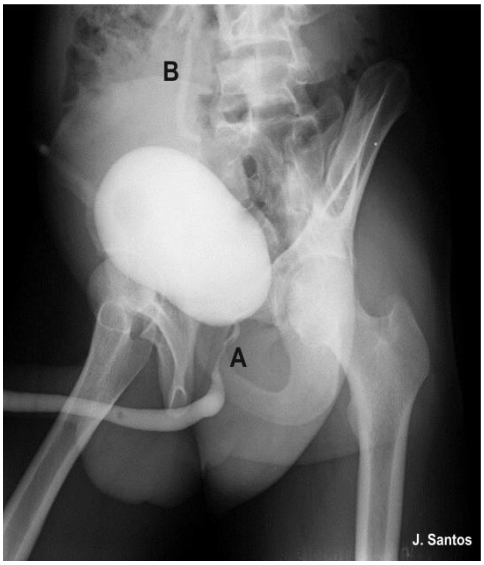

Figura 6. Uretrocistografia: A. estreitamento incompleto da ureta; B. refluxo vesico ureteral (Original)

A Urografia de Eliminação (UE), em casos clínicos com manifesta uropatia obstrutiva, revela dilatação pielocalicial e o nível da obstrução. A uretrocistografia pode demonstrar a presença de refluxo vesicoureteral, o que acorre em 25% dos casos que envolvem os ureteres (Lehman et al., 1973; Smith, 1974) (Figura 6). No entanto, a ecografia ou ultrassonografia (US) é um exame de excelência e não invasivo para o diagnóstico da patologia orgânica relacionada com a UGS, e é particularmente útil para avaliar a evolução após terapêutica e/ou após interrupção da exposição à infeção (Ritcher, 2000). O exame endoscópico e ecográfico, são de elevada importância na identificação e caracterização das lesões do foro urológico (OMS, 2002). A ecografia revela lesões da parede vesical como hiperecogenicidade, espessamento e irregularidades da mucosa, presença de pseudopólipos e massa, a nível renal, pode observar-se dilatação pielocalicial e ureteral secundária a lesões vesicais causadas por *S. haematobium* (Richter, 2000) (Figura 7). A cistoscopia é um exame endoscópico direto que permite visualizar a uretra e a bexiga. Permite ainda a realização da biopsia e subsequentes estudos histológicos pertinentes. As principais alterações visíveis na UGS são a hiperemia e presença de granulomas periovulares na submucosa da parede vesical (Figura 8). Em síntese, o diagnóstico imagiológico é útil, e cada vez mais consensualmente recomendado.

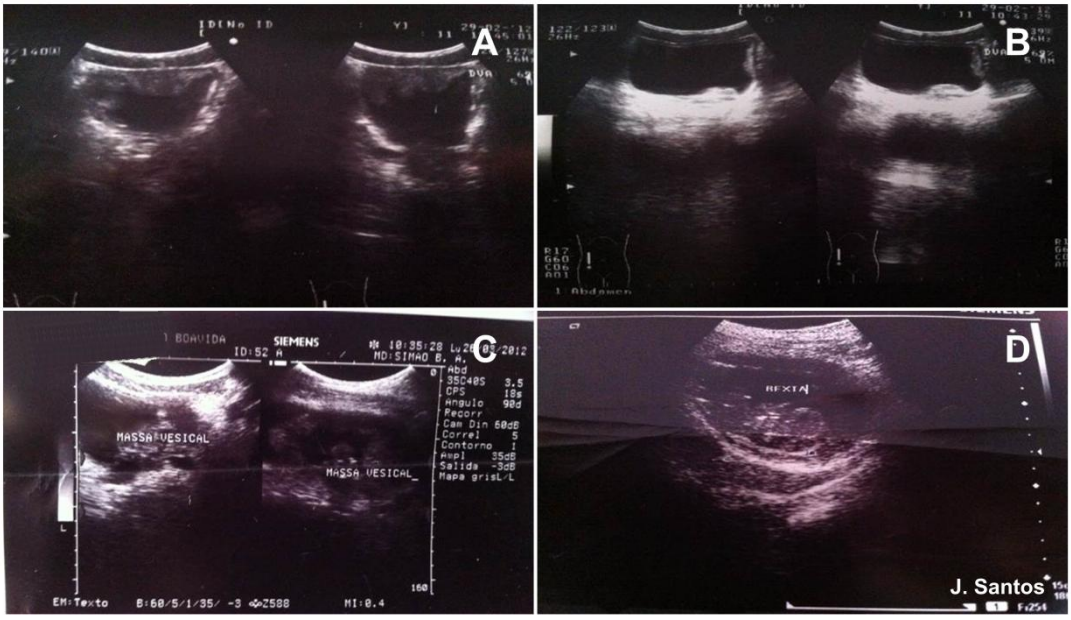

Figura 7. Exames ecográficos: A, parede vesical engrossada; B, pseudopólipo; C e D, tumor vesical. (Original)

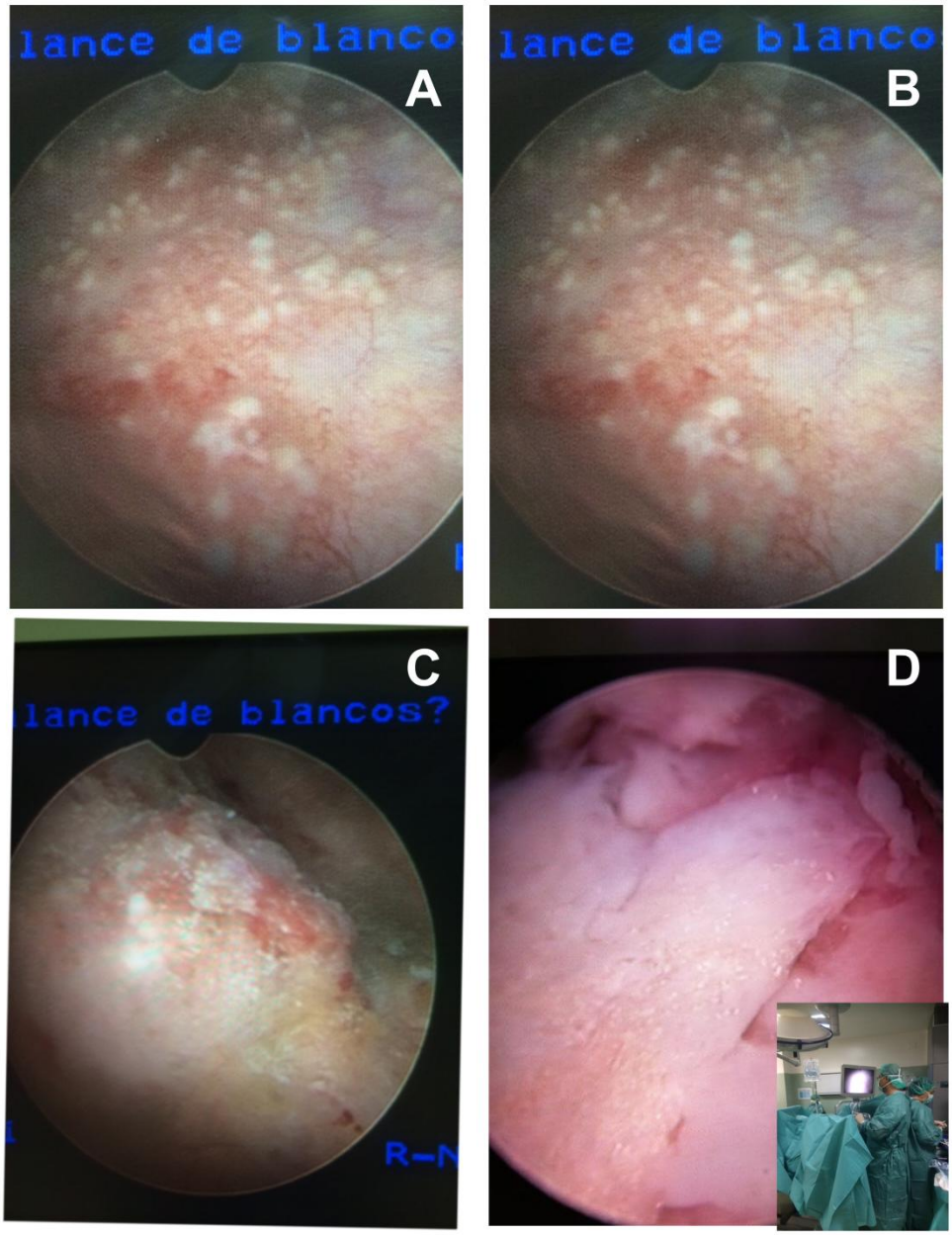

Figura 8. Exames de cistoscopia com lesões de UGS. A e B lesões “sandy patches” e granulomas periovulares; C, granulomas, úlceras e tumor. D, tumor, execução de cistoscopia. (Original)

1.1.10 O Tratamento

O praziquantel (PZQ) (Biltricide® - derivado de pirazinoisoquinolina), é o fármaco de escolha na quimioterapia antiparasitária; eficaz em dose única, 40 mg/kg de peso corporal. No tratamento da UGS permite uma redução da carga parasitária de 95-98% (Mott *et al*. 1985; Ross, 2002; Tchuenté *et al*., 2004; OMS 2006). Num estudo clínico recente realizado no Gana, foi observado que, para além da diminuição da carga parasitária, o tratamento com PZQ diminui, também significativamente, alguns sinais de

indicadores de schistosomose urogenital como: a hematúria macroscópica e microscópica e a proteinúria (Mott et al., 1985). Alguns autores sugerem a existência de fenómenos de resistência parasitária ao PZQ (Greenberg, 2013)). Não tem havido consenso neste assunto. O PZQ continua a ser o recurso terapêutico de eleição.

1.2 *Schistosoma haematobium*, carcinogéneo do grupo I.

A Agência Internacional para Investigação sobre Cancro (IARC) da OMS reconhece a infeção pelo *S. haematobium* como causa definitiva de cancro. Assim, esta infeção, além de comprometer diretamente o desenvolvimento, a saúde e a prosperidade das populações infetadas, propicia o desenvolvimento de carcinoma das células escamosas (SCC no acrónimo inglês; *scamous cells carcinoma*) ou carcinoma espinocelular da bexiga (IARC/OMS, 2012). Este reconhecimento é extensivo a outros agentes infeciosos, nomeadamente Helmintos (*Opisthorchis viverrini* e *Clonorchis sinensis*), com grande expressão clínica e de Saúde Pública em países em vias de desenvolvimento. Estima-se, pois, nestas áreas do mundo, que mais de 20% dos cancros tenham origem infeciosa. Das 5 espécies antropófilas do género *Schistosoma*, apenas o *S. haematobium* foi classificado como carcinogéneo do grupo 1 (IARC/OMS). Dos mais de 110 milhões de casos clínicos de UGS na África subsariana, 70 milhões estão associados com hematúria, 18 milhões com patologia de parede bexiga e 10 milhões com hidronefrose e graves danos renais (van der Werf MJ et al., 2003; King, 2010). O depósito contínuo de ovos de *S. haematobium* na bexiga pode ocasionar carcinoma espinocelular também designado como carcinoma epidermoide (Lopes C.1985; Hodder SL et al., 2000; Parkin DM, 2006). Ferguson em 1911 no Egipto foi o primeiro autor a postular que a transformação maligna associada à UGS seria desencadeada pela irritação prolongada do epitélio da bexiga pela passagem e aprisionamento de ovos. Estes, sabemos hoje, e já o referimos, produzem metabólitos que facilitam a sua progressão através dos tecidos, incluindo a parede da bexiga, alcançarem o lúmen vesical e, posteriormente, pela urina saírem para o ambiente externo e prosseguirem a reprodução da espécie. Cerca de metade dos ovos ficam sequestrados na mucosa vesical originando granulomas periovulares. Tais fenômenos conduzem a hematúria, inflamação, displasia e hiperplasia uroteliais, e carcinoma espinocelular (SCC) (Honeycutt J et al., 2014). A UGS é uma infeção crônica; os vermes adultos sobrevivem muitos anos, as reinfeções ocorrem com frequência e, não raro, o cancro da bexiga associado, ocorre na segunda e terceira décadas de vida (Mostafa MH et al., 1999).

1.3 O SCC e seus modelos etiológicos

O carcinoma espinocelular é uma neoplasia pouco diferenciada, e apresenta-se como a forma mais comum de cancro da bexiga na África rural, onde a UGS é prevalente, e contrasta com a maioria das formas de cancro da bexiga prevalentes nos países desenvolvidos e regiões não endêmicas de UGS, o carcinoma de células transicionais (TCC no acrónimo inglês, *transitional cells carcinoma*), hoje frequentemente designado como carcinoma urotelial. Vários modelos têm sido propostos para explicar a génese de cancro da bexiga induzida pela UGS (Honeycutt J et al., 2014). Alguns atribuem a iniciação da carcinogénese a baixas doses de nitrosaminas e/ou de outros agentes cancerígenos ambientais, associados à infeção (Hicks RM et al., 1980). Noutros modelos, é sugerida a exposição ao fumo do tabaco, a corantes industriais e agrícolas e deficiência de vitamina A (Mostafa MH et al., 1999; Honeycutt J et al., 2014). No entanto, o mecanismo pelo qual a infeção contribui para carcinogénese está ainda por resolver (Rosin MP et al., 1994). Contributos recentes sugerem um papel determinante do *S. haematobium*: células CHO (*Chinese Hamsters Ovary cells*) tratadas experimentalmente com antigénios do parasita evidenciam proliferação, migração e invasão celular aumentadas, apoptose diminuída, a expressão de *Bcl-2* aumentada e a de *p27* diminuída (Botelho M et al., 2009 e 2013) – todos processos biológicos característicos da tumorigénese e sobrevivência da célula tumoral (Hanahan D & RA Weinberg, 2011). Mais ainda, a administração intravesical, num modelo murino, de antigénios de *S. haematobium*, induz displasia urotelial (Botelho M et al., 2010). Estas evidências sugerem que a infeção por *S. haematobium* induz malignização do urotélio, mesmo na ausência de nitrosaminas. Tendo por base estas evidências científicas, e a descoberta de metabólitos de estrogénio de origem parasitária, concatenando moléculas altamente oxidantes do ADN como os catecol estrogénios, moléculas de aductos depurinantes, resultantes da oxidação do ADN do hospedeiro, e moléculas indicadoras de dano genético como 8-oxo-desoxiguanosina, foi postulado um papel para estes metabólitos como iniciadores da carcinogénese (Correia da Costa et al., 2014) (Capítulo 6).

1.4 Os metabólitos de estrogénio e a carcinogénese

Ettore Cavalieri, Eleanora Rogan e colaboradores, 1997, 2002 e 2012, do *University of Nebraska Medical Center* enfatizam o papel dos estrógenos como iniciadores do cancro, num processo genericamente designado de carcinogénese química. De acordo com os seus resultados, metabólitos específicos de estrogénio endógeno, particularmente o catecol estrogénio-3,4-quinona, reagem com o ADN para formar aductos. A posterior libertação destes aductos origina sítios apurínicos (ausência de base azotada) na cadeia de ADN, favorecendo a ocorrência de mutações que podem levar ao início do cancro. O

desequilíbrio no metabolismo dos estrogénios, propiciando a formação excessiva de grupos catecol e de aductos de ADN, aumenta o risco de cancro (ver Capítulo 6). De acordo com os mesmos autores, tais compostos são o fator crítico no início do cancro da mama, da tiroide e do ovário nas mulheres, e entre os homens, o cancro da próstata e o linfoma não-Hodgkin. A formação metabólica de estrogénios deriva da testosterona catalisada por uma aromatase (CYP19), enzima integrante do complexo Coenzima P450 para produzir Estradiol (E2) e Estrona (E1). E2 e E1 são interconvertidos por ação da 17β-hidroxi-estradiol desidrogenase. As oxidases CYP1A e CYP3A catalisam a hidrolisação de E2 originando duas formas de catecol estrogénios, 2-OHE1 (E2) e 4-OHE1 (E2). Estes compostos são inativados por conjugação de glicuronídeos e sulfatos especialmente no fígado. Em tecidos extra hepáticos a sua inativação é mais frequentemente obtida por metilação catalisada pela Catecol-O-metiltransferase (COMT) ou em conjugação com o glutatião por ação da glutatião-s-transferase (GST). Evidências científicas recentes sugerem que os mecanismos metabólicos agora descritos, e relativos aos estrogénios endógenos e ao risco de cancro, estão amplificados durante a infeção por *S. haematobium*; por razões desconhecidas o parasita metaboliza compostos de estrogénio; com frequência, no perfil hormonal de pacientes com UGS o estradiol está aumentado (Botelho et al., 2009); não por produção humana; antes por ação parasitária (Gouveia et al., 2015). Sabemos hoje que o genoma do *S. haematobium* codifica enzimas que poderiam converter progesterona e pregnenolona em estrona, estriol e testosterona. E, curiosamente, foi descrito um gene que codifica um ortólogo da 17β-hidroxi-estradiol desidrogenase (IARC, 2012; Zhou et al., 2009). Tais achados fortalecem a convicção de um metabolismo próprio dos estrogénios nos parasitas que importa conhecer e perceber como iniciadores putativos da carcinogénese associada à UGS.

1.5 Atualização do tema de trabalho e definição de objetivos.

A schistosomose, no sentido clínico lato do termo, é uma doença tropical negligenciada e é considerada a doença humana por helmintos mais importante em termos de morbidade e mortalidade (Hotez et al., 2008; Colley et al., 2014). Estratégias de controlo diversas têm sido desenvolvidas no sentido de bloquear a transmissão e reduzir a carga dessa doença; inclui-se a quimioterapia em massa, melhorias no saneamento, ordenamento do ambiente, ou sequer, o uso de molusquicidas (Colley et al., 2014; Secor et al., 2014). No entanto, a schistosomose, particularmente aquela que nos ocupa, a UGS, continua a ser um problema de saúde pública, especialmente nas regiões rurais da África subsariana (Hu et al., 2004). Além disso, o cancro de bexiga é uma complicação frequente e grave da UGS (Mostafa et al., 1999]. Com efeito, a Agência Internacional de Investigação do Cancro da Organização Mundial de Saúde (IARC/OMS) classifica a infeção por *S.*

haematobium como causa definitiva de cancro, i.e., carcinogéneo do grupo 1 (Hotez et al., 2008; Brindley et al., 2013). A gravidade e a frequência das sequelas da UGS e suas complicações, incluindo carcinoma de células escamosas da bexiga, estão relacionados com a carga e a duração da infeção (Mostafa et al., 1999; Porta et al., 2011). Metabólitos de estrogénio, nomeadamente na sua forma molecular como catecol e de aductos de ADN, estão implicados, pelo menos em parte, nos mecanismos da carcinogénese induzida pela UGS (Correia da Costa et al., 2014) (ver Capítulo 6). Foi nosso propósito, na presente tese, perceber como são sintetizados os metabólitos de estrogénio pelo parasita, como circulam e são excretados durante UGS (Gouveia et al., 2015) (ver Capítulo 4). As causas e consequências da formação destes metabólitos derivados de estrogénio, o modo como promovem danos no ADN e no urotélio dos hospedeiros, permanecem obscuros, e mereceram a nossa atenção. Definimos previamente o nosso trabalho, em parte, como um exercício de aductómica. Utilizamos esta expressão, um neologismo recentemente referido pelo NIH dos USA, para designar o estudo de metabólitos de estrogénio, e de outros compostos químicos indicadores de dano genético no hospedeiro e excretados na urina. Fizemo-lo em Angola, onde reconhecidamente a UGS é um tormento das populações, e onde é urgente intervir. Fizemo-lo num grupo bem caracterizado de doentes com UGS, no seu interesse, e com grande apoio institucional. Que novos metabólitos de estrogénio são formados durante a UGS e como são excretados na urina? Se estes metabólitos promovem dano genético deveremos ser capazes de encontrar sinais moleculares das lesões cromossómicas, nomeadamente a presença de 8-oxo-2'-desoxiguanosina (8-oxodG). Como é excretada esta molécula na urina dos pacientes com UGS? Finalmente, a infeção por *S. haematobium*, variando com o tempo e a intensidade da mesma, induz lesões no urotélio. No que se refere à evolução para cancro, as lesões inicialmente benignas ou pré-malignas evoluem para lesões malignas. Vários autores enfatizam aqui a importância da oncoproteína p53. O gene da p53, *TP53*, está localizado no cromossoma 17p 13. Este gene é alvo frequente de mutações envolvidas na progressão dos tumores (Chaudhry et al., 1997; Elias et al., 2014). Por agora, não é este o aspeto fundamental das nossas preocupações. Antes o seu papel biológico. Funções diversas para a p53 têm sido descritas por diversos autores; o controlo do ciclo celular, a reparação do ADN, a ativação da apoptose (Shaker et al., 2011), a inibição do crescimento tumoral, a supressão de transformação celular (Sun et al., 1996) e manutenção da integridade do genoma (Liu et al., 2008; Nikoletopoulou et al., 2013). A perda de atividade da p53 acelera a tumorigénese e altera a resposta celular a agentes que danificam o ADN (Elias et al., 2014). Quisemos investigar, neste domínio, o que acontece com os nossos doentes. E os resultados são francamente animadores. Previamente, neste exercício, quisemos perceber que tipo de

carcinoma da bexiga está associado à UGS em Angola; procedemos assim a uma análise retrospectiva de 145 doentes internados no Serviço de Urologia do Hospital Américo Boavida em Luanda. Quisemos ainda contribuir para a melhoria da monitorização clínica nos serviços de saúde da Angola profunda. Nesse sentido, são encorajadores os nossos resultados sobre a utilização da ultrassonografia. Disto daremos conta nos capítulos que se seguem.

Capítulo 2

Schistosomose haematobium e carcinoma da bexiga: Análise retrospectiva de 145 doentes internados no Serviço de Urologia do Hospital Américo Boavida em Luanda, in _Acta Urológica Portuguesa._

CAPÍTULO 2

Schistosomose *haematobium* e carcinoma da bexiga: Análise retrospectiva de 145 doentes internados no Serviço de Urologia do Hospital Américo Boavida em Luanda, in *Acta Urológica Portuguesa*.

Júlio Santos, Jacinta Chaves, Manuel Videira, Mónica Botelho, José Manuel Correia da Costa, Jorge Oliveira, Lúcio Lara Santos.

O artigo apresentado como Capítulo 2 tinha um propósito: conhecer as características epidemiológicas, clínicas e patológicas dos doentes com neoplasia maligna da bexiga internados no Serviço de Urologia do Hospital Américo Boavida em Luanda (HAB) e eventual associação com a infecção por S*chistosoma haematobium*. O estudo incidiu sobre 145 doentes com diagnóstico de neoplasia maligna da bexiga admitidos e tratados entre Janeiro de 2006 e Dezembro de 2009. Na série estudada, 102 doentes eram do sexo feminino e 43 do sexo masculino. A idade mediana foi de 47 anos (mínimo 15 anos e máximo 75 anos). A hematúria associada a cistalgia, disúria e anemia foram os sinais e sintomas mais referidos; os dados epidemiológicos revelaram que 48% dos doentes tinham tido contacto com água potencialmente contaminada com S. *haematobium*. Foi comprovada esta infeção em 48,9% dos doentes. Os relatórios anatomopatológicos após biópsia relativos a 107 doentes referiam que 12 apresentavam papilomas uroteliais, e 95, carcinomas da bexiga. O carcinoma espinocelular da bexiga foi descrito em 58,8% dos casos. Na sua maioria, os doentes apresentavam neoplasias localmente avançadas, disseminada ou não tinham condições clínicas para suportarem tratamento cirúrgico radical. A infeção por S. *haematobium* coexiste na maioria dos casos de cancro da bexiga admitidos no HAB em Luanda – Angola, e o carcinoma espinocelular é dominante.

Schistosomose *haematobium* e carcinoma da bexiga: Análise retrospectiva de 145 doentes internados no Serviço de Urologia do Hospital Américo Boavida em Luanda

Schistosomiasis haematobium *and bladder cancer: Retrospective analysis of 145 patients admitted to the Urology Department at the Américo Boavida Hospital, Luanda*

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Resumo

Introdução: Estudar as características epidemiológicas, clínicas e patológicas dos doentes com o diagnóstico de neoplasia maligna da bexiga internados no Serviço de Urologia do Hospital Américo Boavida em Luanda (HAB) e a eventual coexistência com a infecção por *schistosoma haematobium.*

Materiais e Métodos: Estudo retrospectivo de 145 doentes com o diagnóstico de neoplasia maligna da bexiga admitidos e tratados de Janeiro de 2006 a Dezembro de 2009.

Resultados: Na série estudada 102 doentes eram do sexo feminino e 43 do sexo masculino. A idade mediana foi de 47 anos (mínimo 15 anos e máximo 75 anos). A hematúria associada à cistalgia, disúria e anemia foram os sinais e sintomas mais referidos. 48% dos doentes tinham tido contacto com água potencialmente contaminada com *schistosoma haematobium*, tendo sido comprovada a existência de *schistosoma haematobium* em 48,9% dos doentes. Foi realizada biopsia em 107 doentes, 12 dos quais apresentavam papilomas uroteliais e 95 de carcinomas da bexiga. O carcinoma espinocelular da bexiga ocorreu em 58,8% dos casos. Na sua maioria, os doentes tinham neoplasias localmente avançadas, disseminada ou não tinham condições clínicas para suportarem tratamento cirúrgico radical.

Conclusões: O *schistosoma haematobium* coexiste na maioria dos casos de cancro da bexiga admitidos no HAB em Luanda - Angola. A associação observada e o estádio avançado da doença oncológica no momento do diagnóstico implica acções para o controlo desta endemia e de diagnóstico precoce do cancro da bexiga.

Palavras-chave: Cancro, bexiga, *Schistosoma Haematobium*, Angola.

Abstract

Aims: *To study the epidemiological, clinical and pathological characteristics of patients with bladder neoplasm admitted in the Urology Department at Américo Boavida Hospital, Luanda (ABH) and the coexistence of* Schistosoma haematobium *infection.*

Material and Methods: *A retrospective study of 145 patients with bladder neoplasm admitted and treated in January 2006 to December, 2009.*

Results: *In the series, 102 patients were women and 43 men. The median age was 47 years (15-75 years). Hematuria, cystalgia, dysuria, and anemia were the most reported signs and symptoms and 48% of patients had previously contact with contaminated water.* Schistosoma haematobium *was observed in 48.9% of patients. In 107 patients, after pathological confirmation, 12 were urothelial papilloma and 95 were bladder cancer. Squamous cell carcinoma of the bladder occurred in 58.8% cases. Most of the patients had locally advanced cancer disease, disseminated or performance status, were unable to achieve a radical surgery.*

Conclusions: *Schistosoma haematobium coexist in most cases of carcinoma of the bladder in ABH, Luanda - Angola. The advanced stage of malignancy at diagnosis requires actions to control this endemic disease and early diagnosis of bladder cancer.*

Keywords: *Bladder, cancer, Schistosoma Haematobium, Angola.*

Introdução

A *shistosomose haematobium* é endémica em várias regiões de África e do Médio Oriente, incluindo Israel, Egipto, Síria, Iraque e Irão (figura 1). O *schistosoma haematobium* é um parasita cujo desenvolvimento ocorre em duas fases: uma no organismo humano (o hospedeiro definitivo) e outra no interior de um caracol (*Bulinus* – hospedeiro intermediário) este caracol vive em pequenas porções de águas paradas ou de fraca corrente, e neste é o local onde ocorre a infecção de humanos[1]. As pessoas que necessitam de água doce dos rios para beber, cozinhar, produzir alimentos, lavar a roupa e tomar banho, podem estar em risco de serem infectados pelo *schistosoma haematobium* uma vez que as cercárias podem penetrar na pele nua e intacta que está dentro da água. Já no homem, as cercárias alcançam a corrente sanguínea, passando pelos pulmões, coração até chegar ao plexo sanguíneo da bexiga. Este parasita induz irritação crónica e inflamação na bexiga e pode promover condições para o aparecimento de lesões pré-malignas, e a transformação maligna do urotélio[1,2]. Existem evidências epidemiológicas que sugerem a associação entre a infecção por *schistosoma haematobium* e o aparecimento do cancro da bexiga[1,2]. Recentemente Botelho MC e colaboradores demonstraram que células uroteliais normais, quando expostas ao antigénio total deste parasita, aumentavam a sua taxa de proliferação, e que em modelos murinos, bexigas expostas ao mesmo antigénio desenvolvem lesões displásicas, sugerindo que este antigénio pode estar associado ao processo de cancerização[3,4].

Figura 1) Distribuição do *Schistosoma haematobium* (Angola é um país com elevada prevalência)

Figura 2) Ovo de *Schistosoma haematobium*

O grau de infecção pelo *schistosoma haematobium* tem aparentemente um papel importante na indução de diferentes tipos de carcinoma da bexiga. O carcinoma espinocelular está geralmente associado a uma carga parasitária moderada a alta, enquanto o carcinoma urotelial ocorre mais frequentemente em áreas associadas a menores taxas de infecção[5]. Apesar das suas consequências, esta doença tropical é hoje, infelizmente, uma das doenças tropicais negligenciadas[6].
Figueiredo J e colaboradores ao estudarem 300 indivíduos provenientes de uma região de Angola onde a *schistosomose haematobium* é endémica verificaram que 71,7% encontravam-se infectados[7].
Luanda e os agrupamentos populacionais limítrofes têm elevada prevalência de *schistosoma haematobium*, sendo por isso espectável que o carcinoma da bexiga, diagnosticado nesta região, esteja maioritariamente associado à infecção dos doentes por este parasita. Para avaliar esta hipótese e definir um plano de acção, decidiu-se estudar as características epidemiológicas, clínicas, patológicas e estádio da doença dos casos admitidos no Serviço de Urologia do Hospital Américo Boavida de 2005 a 2009, com o diagnóstico de neoplasia da bexiga.

Material e Métodos

De acordo com o registo de doentes internados do Serviço de Urologia do Hospital Américo Boavida em Luanda, entre Janeiro de 2005 e Dezembro de 2009, foram internados 1882 doentes, dos quais 145 (7,7%) com a suspeita clínica de neoplasia da bexiga. Após a obtenção de autorização por parte das autoridades hospitalares e da comissão de ética, e através de análise de informação registada nos processos clínicos, avaliaram-se, retrospectivamente as características epidemiológicas, clínicas, anatomo-patológicas, estádio da doença, tratamento realizado e a evolução destes doentes. As variáveis sexo, idade, residência, naturalidade, factores de risco, sintomas e sinais, diagnóstico histológico, exames auxiliares de diagnóstico realizados, complicações associadas à doença, tratamento médico e cirúrgico, tempo de internamento e estado no momento da alta, foram objecto de registo em formulário específico. A classificação histológica, estádio e tratamento foi realizado de acordo com as recomendações da Associação Europeia de Urologia[8,9]. A análise dos dados foi feita utilizando o programa Statistical Package for Social Sciences (SPSS) versão 17.0®. As variáveis estudadas foram apresentadas em tabelas de frequências e histogramas. Para estudar a associação entre o género (f/m) e variáveis categóricas estudadas utilizou-se o teste do Qui-Quadrado. A comparação da média de idade, por género, foi realizada com recurso à análise de variância simples. A análise estatística teve em conta um nível de significância inferior a 0,05.

Resultados

No Serviço de Urologia do Hospital Américo Boavida em Luanda são internados cerca de 30 doentes com carcinoma da bexiga por ano. Dos 145 doentes com suspeita clínica de carcinoma da bexiga estudados, 102 (70%) eram do sexo feminino e 43 (30%) do sexo masculino sendo a relação homem:mulher de 1:2,3. A idade mediana foi de 47 anos (mínimo 15 anos e máximo 75 anos) (figura 3). A distribuição por grupos etários e a média de idades, segundo o género, foi sobreponível não havendo diferenças estatisticamente significativas. Não observámos diferenças significativas nas variáveis epidemiológicas e clínico-patológicas estudadas, no estádio da doença, na terapêutica realizada e na evolução clínica, quando comparámos os diversos grupos etários e o género (f/m). Em relação a factores de risco verificou-se que 71 (48%) doentes tinham tido contacto com água potencialmente contaminada com *schistosoma haematobium*. A tabela I apresenta o resumo de dados relativos às variáveis clínicas e patológicas estudadas. A hematúria associada à cistalgia, disúria e anemia foram os sinais e sintomas mais frequentemente referidos e motivo de internamento em ambos os sexos. A ecografia pélvica e a cistoscopia foram os exames auxiliares de diagnóstico mais vezes utilizados. Apenas em 107 (73,7%) doentes da amostra foi possível obter a caracterização histológica da neoplasia da bexiga. Foram diagnosticados 12 papilomas uroteliais e 95 de carcinomas da bexiga. O tipo histológico mais frequente foi o carcinoma espinocelular da bexiga em 63 (58,8%) casos. Nos doentes em que não foi realizada biopsia, o diagnóstico de neoplasia da bexiga foi obtido com recurso à citologia urinária aliada a exames de imagem e história clínica, estes doentes tinham doença avançada, complicações e mau estado geral.

Comprovou-se a existência de *schistosoma haematobium* em 71 (48,9%) doentes da amostra. Destes, em 6 doentes foram observados ovos do parasita na urina, em 25 doentes os ovos foram observados na mucosa vesical durante a cistoscopia e nos restantes 40 doentes existiam ovos de *schistosoma haematobium* nas peças cirúrgicas e nas biopsias realizadas para confirmar o diagnóstico de carcinoma da bexiga.

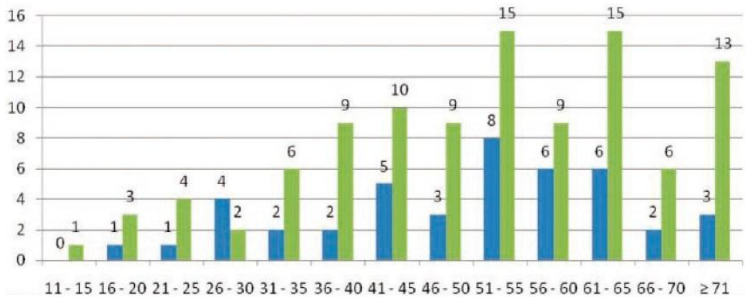

Tabela I) Perfil de resistência dos microorganismos produtores de ESBL

Na sua maioria, os doentes tinham neoplasias localmente avançadas (T3/T4) ou não tinham condições clínicas para suportarem tratamento cirúrgico radical. Assim, apenas 5 (3,4%) doentes foram submetidos a tratamento cirúrgico com intenção curativa (3 – ressecções trans-uretrais e 2 cistectomias parciais). Dezoito doentes foram submetidos a tratamento cirúrgico das complicações e os restantes 122 doentes tiverem tratamento paliativo. Dos doentes avaliados, 5 (3,4%) tiveram alta livres de doença, 53 (36,5%) mantiveram cuidados paliativos e 77 (53,1%) faleceram durante o internamento.

Variável	Nº de casos	Percentagem
Género*		
Masculino	43	29,6%
Feminino	102	70,4%
Factores de Risco*		
Contacto com água contaminada com *Schistosoma haematobium*	71	48,8%
Tabagismo	44	30,3%
Indústria de Risco	11	7,5%
Desconhecido	19	13,1%
Sintomatologia Dominante*		
Hematúria	132	91,0%
Cistalgia	101	69,6%
Piúria	90	62,0%
Disúria	80	55,1%
Retenção urinária aguda	30	20,6%
Meios auxiliares de Diagnóstico*		
Ecografia abdomino-pélvica	131	90,3%
Cistoscopia com biopsia	123	84,8%
Raio-X da bacia	110	75,8%
Urografia intravenosa	21	14.4%
TC abdomino-pélvica	20	13,7%
Complicações associadas*		
Anemia	138	95,1%
Insuficiência renal aguda	33	22,7%
Sépsis (urinária)	15	10,3%
Hidronefrose	13	8,9%
Fistula vesico-cutânea	3	2,0%
Fistula vesico vaginal	1	0,6%
Tipos histológicos**		
Papiloma urotelial	12	11,2%
Carcinoma papilar de baixo grau	9	8,4%
Carcinoma *in situ*	4	3,7%
Carcinoma invasor espinocelular	63	58,8%
Adenocarcinoma invasor	18	16,8%
Outras neoplasia malignas invasoras	1	0,9%

Tabela I) Variáveis epidemiológicas, clínico-patológicas e terapêuticas
* em relação aos 145 doentes;
** em relação as 107 doentes em que foi possível realizar biopsia.

Discussão

O presente estudo pretendeu avaliar o perfil dos doentes com cancro da bexiga admitidos e tratados no Serviço de Urologia do Hospital Américo Boavida em Luanda – Angola, a sua eventual associação à infecção por *schistosoma haematobium* e em função dos resultados definir linhas de acção.

O pico de incidência de cancro de bexiga, nos países ocidentais ocorre na sexta ou sétima década de vida e apenas 12% dos casos ocorrem em pessoas com menos de 50 anos[10-12]. O tipo histológico predominante é o carcinoma urotelial e o tabagismo está associado ao processo de desenvolvimento de cancro da bexiga. Contrastando com estes dados nos países em que a *schistosomose haematobium* é endémica, a idade média dos casos de cancro da bexiga varia entre os 40 e 49 anos[2,13-15]. Em relação ao género, a relação homem:mulher nesses países, varia entre 4:1 e 5,9:1, sendo esta relação explicada pelo facto de nas áreas rurais a via de infecção ocorrer através do contacto com águas infectadas, durante as actividades agrícolas, sendo estas, normalmente, realizadas por homens[16]. Observámos que a idade mediana na série estudada é semelhante à descrita para áreas de elevada prevalência de *schistosomose haematobium* porém a relação homem:mulher diverge dos dados publicados, uma vez que o género feminino é prevalente. Esta diferença pode ser explicada pelo facto de que em Angola, a população economicamente activa e que se dedica à actividade agrícola ser maioritariamente do sexo feminino[17]. Corroborando esta hipótese Figueiredo J e colaboradores verificam que a infecção por *Schistosoma haematobium* em indivíduos que viviam na região de Luanda era mais frequente em jovens e na população feminina[7]. Este estudo permitiu demonstrar a coexistência da infecção por *schistosoma haematobium* numa elevada percentagem dos doentes admitidos com a suspeita clínica de cancro da bexiga (48,9%). Adicionalmente, o facto de o tipo histológico mais frequentemente observado ser o carcinoma espinocelular, realça a importância desta endemia na génese do cancro da bexiga na população admitida e tratada no Hospital Américo Boavida. O tratamento cirúrgico é eficaz quando a neoplasia é restrita à bexiga. Porém na amostra estudada, os doentes na sua maioria tinham tumores localmente avançados e associados a complicações importantes, sendo apenas possível tratamento paliativo, o estádio da doença no momento do diagnóstico foi responsável pela elevada taxa de mortalidade observada.

Este cenário sublinha a necessidade de acções de prevenção primária, onde o tratamento precoce da infecção é capital, bem como o diagnóstico precoce da doença oncológica.

Segundo a Organização Mundial de Saúde (OMS), mais de 206 milhões de pessoas estão infectadas com parasitas do género *schistosoma* (dos quais 120 milhões têm sintomas e cerca de 20 milhões têm sequelas graves) e mais de 600 milhões de pessoas vivem em áreas endémicas[18].

Locais sentinela, como por exemplo a determinação da taxa de infecção na população escolar em áreas de risco, tem se revelado uma metodologia importante na definição da dimensão do problema nas regiões estudadas[19-21]. A prevalência da infecção por *schistosoma haematobium* na população escolar em distintas localidades da província de Luanda variou entre os 30% e os 45%[22].

Desde 1980, o praziquantel tornou-se disponível e demonstrou ser uma droga eficaz na luta contra o *schistosoma haematobium*. Este medicamento tem efeitos adversos mínimos e baixo custo, sendo por esse motivo um instrumento útil em programas de saúde pública que visem o controlo desta doença[23]. O Ministério de Saúde de Angola tem um programa nacional de controlo da *schistosomose*. Adicionalmente, projectos de educação, formação e investigação estão em curso envolvendo instituições médicas e de ensino[17,22,24]. No entanto este estudo aponta para a necessidade de monitorizar os programas referidos avaliando a sua eficácia e a definição de um protocolo de diagnóstico precoce de cancro da bexiga onde acções bem estabelecidas devem acontecer de imediato perante o diagnostico de hematúria. A experiência internacional advoga que nestas actividades sejam envolvidos a população, as autoridades de saúde, o governo, as universidades e a iniciativa privada no sentido de maximizar o sucesso.

Conclusão

A análise retrospectiva realizada, sugere a existência de uma relação entre a infecção por *schistosoma haematobiume* e o desenvolvimento de carcinoma da bexiga em Luanda - Angola. O estádio avançado da doença oncológica no momento do diagnóstico acentua a necessidade de uma acção coordenada para o controlo desta endemia e de diagnóstico precoce da infecção e da doença oncológica vesical co-relacionada.

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Capítulo 3

Comparison of findings using ultrasonography and cystoscopy in urogenital schistosomiasis in a public health centre in rural Angola, in *South African Medical Journal.*

CAPÍTULO 3

Comparison of findings using ultrasonography and cystoscopy in urogenital schistosomiasis in a public health centre in rural Angola, *in South African Medical Journal*.

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O artigo apresentado como Capítulo 3 tinha um propósito: avaliar a eficácia da ultrassonografia (US), vulgo ecografia, como instrumento de diagnóstico imagiológico na UGS. O exame cistoscópico (CE) tem sido indicado como teste de referência no diagnóstico de manifestações tardias da UGS, incluindo o cancro da bexiga. Contudo, duas imperfeições lhe são apontadas: i) o seu carácter invasivo; ii) a sua baixa sensibilidade (10 a 40% dos tumores podem não ser detetados). Existe alguma urgência e consensualidade na necessidade de definir de métodos de diagnóstico imagiológico mais eficientes e não invasivos, ou ainda, na seleção de uma nova geração de biomarcadores eficientes no controlo da UGS. Tal como descrito, uma coorte de 80 pacientes infectados com *S. haematobium* foi seleccionada a fim de comparar as alterações na parede da bexiga detectadas por US com os observados por cistoscopia. Os resultados observados revelam uma correlação notável nos elementos colhidos por CE e US. Doentes com lesões da mucosa da bexiga, tais como neoplasias, úlceras ou granulomas periovulares observados por CE, revelaram mudanças na espessura da parede da bexiga na observação por US. Os resultados suportam a crescente utilização de ecógrafos portáteis para diagnóstico da UGS, mesmo por médicos de clínica geral que operam no terreno. Consideramos que o exame por US deve ser parte integrante fundamental na investigação de hematúria, nos programas de controlo da UGS, bem como nos programas de formação de Médicos de clínica geral em Angola.

Comparison of findings using ultrasonography and cystoscopy in urogenital schistosomiasis in a public health centre in rural Angola

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Background. Schistosomiasis is a chronic disease caused by infection with parasitic worms of the genus *Schistosoma*. In sub-Saharan Africa, infections with *S. haematobium* are most common. Cystoscopic examination (CE) has been accepted as the gold-standard test for detecting the late manifestations of schistosomiasis, including urothelial cancer of the bladder. However, this procedure is invasive and 10 - 40% of tumours may remain undetected. A non-invasive examination and a new generation of biomarkers are needed for better monitoring of the disease.
Objective. To assess the usefulness of ultrasound (US) scans for monitoring of structural urinary tract disease by local public health services in areas of Angola in which urogenital schistosomiasis is endemic.
Methods. A cohort of 80 *S. haematobium*-infected patients was selected in order to compare changes in the bladder wall detected by US with those observed on CE.
Results. There was a notable correlation between the findings observed on CE and US. Patients with lesions of the bladder mucosa such as neoplasms, ulcers or granulomas detected by CE also had changes in bladder wall thickness on US. The results support increased use of portable US machines for non-invasive examination of the bladder by local general practitioners.
Conclusion. US examination should be an integral part of the investigation of haematuria and used in all *S. haematobium* control programmes. General practitioners may find it useful for more accurate diagnosis of haematuria and to identify bladder wall alterations in both adults and children in schistosomiasis-endemic regions.

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Anthropophilic species of *Schistosoma* currently infect more than 200 million people in 77 countries in the endemic areas of Africa, the Caribbean, Central America, South America, East Asia and the Middle East.[1] More than 90% of these cases occur in Africa, and of these approximately two-thirds are caused by *S. haematobium*.[2] Recently King[3] suggested that the number of cases of *S. haematobium* infection may be greater than was previously believed. If this is confirmed, urogenital schistosomiasis may represent the most common infection in sub-Saharan Africa.[3]

Many infections with *S. haematobium* result in minimal symptoms or cause haematuria, dysuria, anaemia and inflammation of the urinary tract. However, a significant proportion of infected patients, ranging from 25% to 50%, experience moderate to severe morbidity. Late manifestations include kidney dysfunction, ureteric obstruction and squamous cell carcinoma of the bladder. Cystoscopic examination (CE) is accepted as the gold standard for the detection of disorders of chronic schistosomiasis.[4] However, this procedure is invasive and 10 - 40% of malignancies may be undetected. Microscopic examination of the urine remains the gold-standard test for the diagnosis of urogenital schistosomiasis, although it has some limitations. Detection of specific antibodies and/or antigens by serological assays can be useful in specific circumstances in endemic areas, but their application is limited[5] beyond diagnosis of the disease in travellers from non-endemic areas.[6] Schistosomiasis can be controlled with safe and effective drug therapy. Other components of management include the provision of potable water, adequate sanitation, hygiene education and snail control.[6-7]

In a resource-poor setting with numerous limitations, what can general practitioners, other healthcare professionals and local health services do for populations in their care in regions endemic for schistosomiasis? Lesions caused by infection with *S. haematobium* can be detected readily on ultrasound (US) examination. King[3] reported that US-based population surveys enabled effective assessment of reversibility of structural changes in a range of age groups, and provided data assisting choice of treatment protocols.

The present study assessed the performance of US detection of bladder wall changes in a group of patients in a schistosomiasis-endemic area (Pita, Angola; Fig. 1) referred to a public clinic with

nonspecific haematuria, comparing and contrasting changes in the bladder wall detected by US with those observed at CE. To our knowledge, this is the first time such an assessment has been done.

Methods

Study sites and participants

Ethical approval for the study was granted by the ethics committee of Agostinho Neto University, Luanda, Angola. Parental consent in the case of children and consent from participating adults was obtained.

Eighty people were recruited into the study between December 2011 and September 2012. Of these, 70 were consecutive patients with macroscopic haematuria who attended a rural public clinic (Pita-Muxima) in Luanda Province (municipality of Quissama). An additional 10 were admitted to the Department of Urology at Américo Boavida Hospital (ABH), Luanda, with haematuria and suspected bladder cancer. All participants had bladder US scans with subsequent CE and mucosal biopsy.

Urine

Urine samples were collected from the 80 patients. They were tested for haematuria using reagent strips (Hemastix, Bayer, UK). Parasitological examination for detection of *S. haematobium* eggs was done by filtering a 10 ml aliquot through a polycarbonate filter with a pore size of 12 µm diameter (Millipore, UK). The slides were examined at × 100 under a compound light microscope, according to World Health Organization (WHO) guidelines.[8]

Ultrasound examination

Fluid was given to each patient 30 minutes to 1 hour before US examination to ensure adequate filling of the bladder to assess its shape and any wall irregularity. A portable US apparatus (Siemens portable ultrasound with a 3.5 MHz curvilinear probe, WA 98029-7002, USA) was used. The scans were done by medically qualified persons, particular attention being paid to abnormalities of bladder shape, irregularities or thickening of the bladder wall, discernible masses and presence of polyps, calcification and/or hydronephrosis. Findings were recorded photographically. WHO criteria were used to classify any bladder damage detected.[9]

Cystoscopic examination

Patients underwent CE in the Department of Urology at ABH. CE was performed after the US scan, and particular attention was given to major bladder mucosa alterations such as ulcers, schistosome eggs, granulomas and tumours (14 patients had schistosome eggs and other mucosal changes). A Karl Storz cystoscope, 20-Fr (adults) and 11.5-Fr (children), with a 30° eyepiece was used.

Fig. 1. Pita, Luanda Province, Angola.

Treatment

Prazinquantel 40 mg/kg was offered to patients diagnosed with schistosomiasis, according to WHO guidelines.[10] Those who were diagnosed with bladder cancer were admitted to the urology service of ABH for specific treatment.

Statistical analysis

Statistical analysis was performed using SPSS 17 statistics software (SPSS Inc. for Windows). Demographic, bladder US and cystoscopy data were recorded. χ^2 analysis using Fisher's exact test (two-tailed) was performed for categorical data, with a p-value of ≤0.05 considered statistically significant.

Results

The median age of the patients was 41 years (range 3 - 75); 62 (77.5%) were males. All had macroscopic haematuria, and the reagent strips were positive for the presence of blood. A parasite egg count was positive in 36 patients (45.0%). All the paediatric patients were treated for the first time during this study. Bladder wall irregularities detected by US are described in Table 1. In brief, 60 (75.0%) of the participants had distortion of the bladder shape and 29 (36.3%) had bladder masses (Fig. 2). Major findings on CE are set out in Tables 1 and 2.

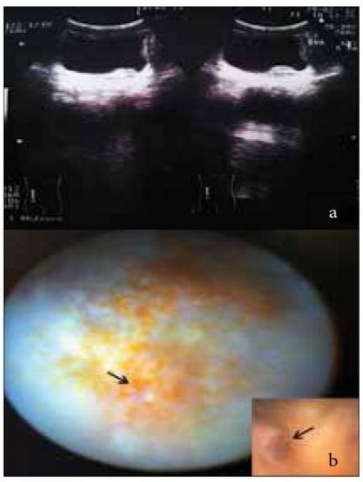

Fig. 2. (a) US bladder wall irregularities and (b) appearance on CE in a patient with haematuria (arrows = granuloma).

Here, 54 patients (67.5%) had only schistosome eggs, granulomas being present in 14 (17.5%). Neoplasms were seen in 10 patients (12.5%) and ulcerations in 2 (2.5%), both of whom were <19 years of age. The median age of patients with tumours was 51 years (range 10 - 68).

There was a notable correlation between the findings on CE and US. Patients with lesions in the bladder mucosa such as neoplasms, ulcers or granulomas detected by

Table 1. Gender, presence of haematuria, bladder US and CE features and findings on examination of the urine

Variables	Patients *n* (%)
Gender	
Female	18 (22.5)
Male	62 (77.5)
Haematuria	80 (100.0)
US bladder shape	
Normal	20 (25.0)
Abnormal	60 (75.0)
US bladder wall irregularities	
<5 mm normal	1 (1.3)
≥5 mm focal	40 (50.0)
≥5 mm multifocal or diffuse	39 (48.7)
US discernible masses	
None	51 (63.7)
Single	13 (16.3)
Multiple	16 (20.0)
US presence of pseudopolyps	
Yes	6 (7.5)
No	74 (92.5)
US calcification	
Yes	75 (93.7)
No	5 (6.3)
Hydronephrosis	
Yes	3 (3.7)
No	77 (96.3)
CE	
S. haematobium eggs	54 (67.5)
Granuloma	14 (17.5)
Ulcer	2 (2.5)
Neoplasm	10 (12.5)
Urine	
S. haematobium eggs	36 (45.0)

CE also had changes in bladder thickness on US. Only one patient in whom CE showed the presence of eggs in the mucosa had a normal US scan (Table 3). Residual urine was observed in 17 patients (21.3%), and bilateral hydronephrosis was found in 3 (3.8%). One patient had an associated kidney neoplasm.

The outcomes achieved by the two diagnostic approaches supported an acceptable correlation for the detection of bladder changes by US and CE. While bladder wall distortion or bladder masses in urogenital schistosomiasis were detected by US, it was not possible to obtain meaningful information about bladder thickness (Fig. 3).

Table 2. Dominant features on bladder CE according to age group

	Patients, *n*				
Age group (years)	*S. haematobium* eggs	Granuloma	Ulcer	Neoplasm	Total
≤19	4	8	2	1	15
20 - 29	12	1	0	1	14
30 - 39	4	3	0	1	8
40 - 49	18	1	0	1	20
50 - 59	8	0	0	4	12
60 - 69	6	0	0	2	8
≥70	2	1	0	0	3
Total	54	14	2	10	80

Table 3. Dominant features on bladder CE and irregularities noted on bladder US

		US discernible masses, *n*			
Cystoscopy features	US bladder wall irregularities	None	Single	Multiple	Total, *n*
S. haematobium eggs	<5 mm – normal	1	-	-	1
	≥5 mm focal	29	3	-	32
	≥5 mm multifocal or diffuse	18	2	1	21
Granuloma	<5 mm – normal	-	-	-	-
	≥5 mm focal	1	-	1	2
	≥5 mm multifocal or diffuse	1	2	9	12
Ulcer	<5 mm – normal	-	-	-	-
	≥5 mm focal	1	-	-	1
	≥5 mm multifocal or diffuse	1	-	-	1
Neoplasm	≥5 mm focal	-	5	-	5
	≥5 mm multifocal or diffuse	-	-	5	5

Discussion

US is suitable for monitoring schistosomiasis-related pathology of the urinary tract, and is particularly useful in assessing its evolution after therapy. Bladder alterations observed by US have been associated with known biomarkers of bladder cancer in adults with chronic *S. haematobium* infection.[11] The importance of US examination in monitoring structural disorders in urogenital schistosomiasis has long been established. US provides clear evidence of ureteric wall abnormalities causing strictures and ureterocele-like lesions of the ureteric ostia, and demonstrates resolution of bladder lesions after treatment, including in paediatric patients.[12]

A recent report indicates that general practitioners can learn to carry out a simple US examination for accurate diagnosis and follow-up of *S. haematobium* infection-related bladder lesions within a few sessions.[13] The aim of the present study was to verify the reproducibility of the bladder changes detected by US and bladder mucosa lesions observed by CE in order to teach general doctors to perform bladder US as an additional tool in the diagnosis of haematuria in areas endemic for *S. haematobium*.

Our results support other observations on *S. haematobium*-infected patients >30 years of age, in whom severe bladder damage was evident on US examination but no *S. haematobium* eggs were present in their urine samples.[14] The optimal imaging modality for patients with haematuria remains controversial, particularly given developments in computed tomography urography and magnetic resonance urography.[15] However, the European Society of Urogenital Radiology guidelines for the investigation of painless haematuria recommend the use of US.[16]

Our results confirm that in patients with haematuria, the changes seen on US examination of the bladder correlated significantly with the presence of eggs of *S. haematobium*, ulcers or granulomas in the bladder mucosa observed at CE. We consider that a training programme for general practitioners in US for this purpose would be valuable in developing countries. Portable US devices are also useful for the evaluation of breast, gynaecological and urological pathology, and should be among the tools employed for routine examinations by the general doctor in resource-poor settings.[17]

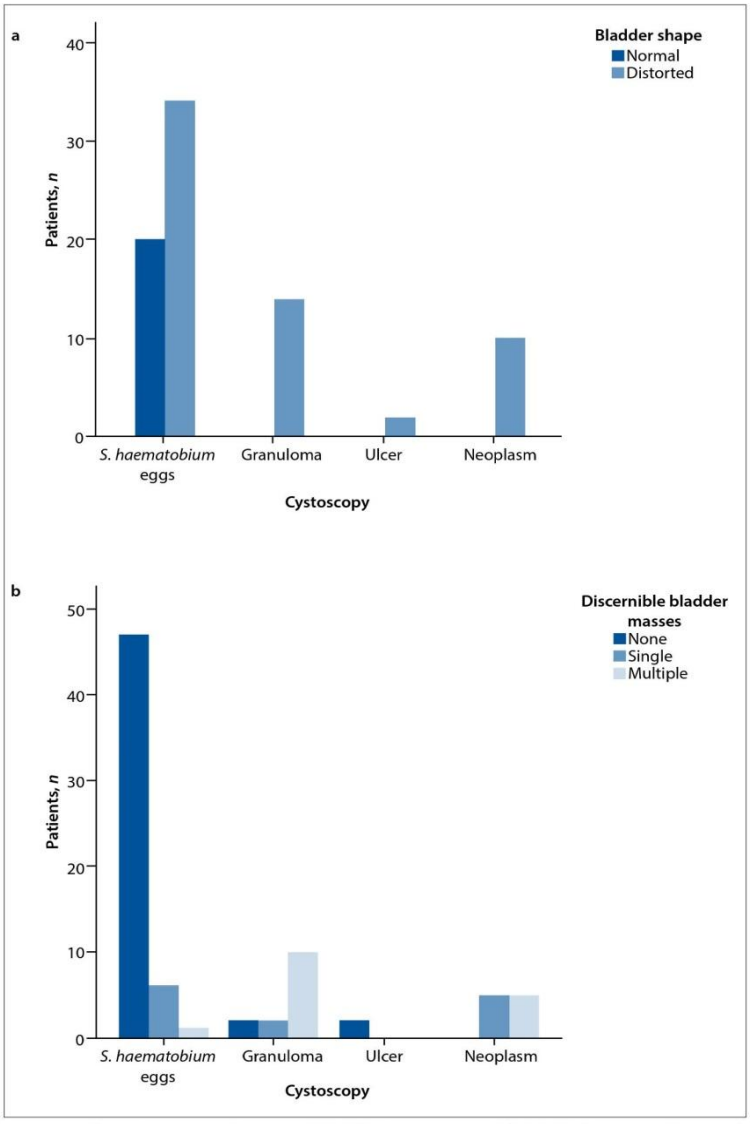

Fig. 3. Correlation between CE features and bladder changes on US: (a) bladder shape on US (p=0.005); (b) masses detected on US (p=0.0001).

Infection with *S. haematobium* has been detected in 64.8% of adults examined in the north of Angola.[18] At the 2014 International Congress of Angolan Physicians in Luanda, Pintar *et al.*[19] presented their finding that 83% of 149 children with *S. haematobium* infection had bladder wall changes that were evident on US.

Angola has a programme of prevention, control and elimination of neglected diseases such as schistosomiasis that includes the training of general practitioners in US to help achieve early diagnosis of urogenital schistosomiasis and to monitor the results of chemotherapy. The introduction of tests of high sensitivity and specificity, such as detection of parasite-specific DNA in urine,[11,20] will be of added value in monitoring elimination programmes.

Conclusions

US examination should be an integral component of the diagnosis of haematuria and should be performed in all *S. haematobium* control programmes in schistosomiasis-endemic regions, including in paediatric populations.

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Author contributions. JS and LLS conceived and designed the experiments, JS, JC and HA performed the experiments, JS, CL, JMC and LLS analysed the data, and JS, CS, JD, JN, NV, CL, PJB, JMC and LLS wrote the paper.

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Capítulo 4

Estrogen-like Metabolites and DNA-adducts in Urogenital Schistosomiasis-associated bladder cancer, in *Cancer Letters.*

CAPÍTULO 4

Estrogen-like Metabolites and DNA-adducts in Urogenital Schistosomiasis-associated bladder cancer, *in Cancer Letters.*

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O artigo apresentado como Capítulo 3 tinha um propósito: encontrar evidências científicas em humanos que permitam a sustentação científica do postulado do nosso grupo que atribui a metabólitos de estrogénio um papel de grande relevância nos mecanismos moleculares implicados na carcinogénese vesical associada a infeção por *S. haematobium* (ver Capítulo 6). Tal postulado sustentava-se em observações verificadas em modelos celulares e animais experimentais. Agora, operando com um grupo de doentes de Angola com UGS, bem caracterizado do ponto de vista clínico, histológico e epidemiológico, colheram-se amostras de urina e tecido da mucosa da bexiga. As amostras de urina foram submetidas a análises por Cromatografia Líquida acoplada a Espectrometria de Massa (LC-MS/MS). Os resultados obtidos enfatizam a existência de um metabolismo parasitário de metabólitos de estrogénio, ainda não completamente entendido, e a excreção urinária de metabólitos específicos dos doentes com UGS, e com um perfil cromatográfico que indicia diferenças substantivas entre doentes com UGS e cancro da bexiga e doentes com UGS sem cancro. Os metabólitos identificados nas amostras de urina incluem moléculas na forma quinona de catecol estrogénio (com grande capacidade de reagir com o ADN) e aductos depurinantes (moléculas resultantes da reação com o ADN e constituídas por um grupo estrogénico associado a uma base azotada retirada ao ADN, usualmente Adenina ou Guanina). Alguns deles foram previamente identificados no verme adulto e nos ovos de *S. haematobium*, Notável foi a observação de uma molécula indiciadora de lesões no ADN: 8-dihidro-desoxiguanosina (8oxodG).

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Original Articles

Estrogen-like metabolites and DNA-adducts in urogenital schistosomiasis-associated bladder cancer

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ABSTRACT

An estrogen-DNA adduct mediated pathway may be involved in the pathogenesis of the squamous cell carcinoma of the bladder associated with infection with the blood fluke *Schistosoma haematobium*. Extracts from developmental stages of *S. haematobium*, including eggs, induce tumor-like phenotypes in cultured cells. In addition, estrogen-derived, reactive metabolites occur in this pathogen and in sera of infected persons. Liquid chromatography-mass spectrometry analysis was performed on urine from 40 Angolans diagnosed with urogenital schistosomiasis (UGS), half of who also presented UGS-associated squamous cell carcinoma and/or urothelial cell carcinoma. The analysis revealed numerous estrogen-like metabolites, including seven specifically identified in UGS cases, but not reported in the database of metabolites in urine of healthy humans. These schistosome infection-associated metabolites included catechol estrogen quinones (CEQ) and CEQ-DNA-adducts, two of which had been identified previously in *S. haematobium*. In addition, novel metabolites derived directly from 8-oxo-7, 8-dihydro-2′-deoxyguanosine (8-oxodG) were identified in urine of all 40 cases of UGS. These metabolites can be expected to provide deeper insights into the carcinogenesis UGS-induced bladder cancer, and as biomarkers for diagnosis and/or prognosis of this neglected tropical disease-linked cancer.

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Introduction

Schistosomiasis is one of the major neglected tropical diseases and it is considered the most important helminthic disease of humanity in terms of morbidity and mortality. More than 90% of the cases occur in Africa, of which about two-thirds are caused by *Schistosoma haematobium* [1–5]. Indeed, the number of cases of infection with *S. haematobium* may far exceed that previously predicted so that urogenital schistosomiasis (UGS) may represent the most common infection or even adverse health condition in sub-Saharan Africa [6]. In addition, female genital schistosomiasis increases the risk of transmission of HIV [7–9], and a recent outbreak in Corsica confirms its re-emergence in Europe [10,11]. Many cases of UGS result ostensibly in only mild symptoms and disease such as hematuria, dysuria, anemia and inflammation of the genital-urinary tract [6,12–16]. However, between 25 and 50% of the UGS cases experience moderate to severe morbidity [6], including renal dysfunction, obstruction of the ureters, and squamous cell carcinoma of the urinary bladder [17–20]. Bladder cancer is a frequent and dire complication of chronic UGS. Case reports indicate that patients with schistosomiasis may develop bladder cancer earlier than uninfected people. The severity and frequency of the sequelae of UGS and of its complications are related to the intensity and duration of the infection [21–23]. Moreover, infection with *S. haematobium* is classified as a Group 1 biological carcinogen by the World Health Organization (WHO)'s International Agency for Research on Cancer (WHO IARC) [24] although the cellular and/or molecular mechanisms linking *S. haematobium* infection with carcinogenesis have yet to be defined [25,26]. It has been known for several decades that bladder cancer, especially squamous cell carcinoma (SCC), a distinctly malignant, poorly differentiated neuroendocrine neoplasm [16], was geographically associated with UGS, i.e. areas endemic for schistosomiasis haematobia [20,24,27]. In regions with high worm burdens, and a high risk of exposure to

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S. haematobium infection, SCC is the most frequent histological type, whereas urothelial cell carcinoma (UCC) is predominant in non-endemic locations [22–24,26,28,29].

An estrogen-DNA adduct mediated pathway in *S. haematobium*-infection associated bladder cancer has been postulated [27,30,31]. We have identified and characterized by liquid chromatography-mass spectrometry (LC-MS/MS) novel estrogen-like metabolites, present in the lysates and secretions of *S. haematobium* worms and eggs [27], and in sera of UGS cases [30]. Moreover, lysates of *S. haematobium* induce tumor-like phenotypes; Chinese Hamster Ovary cells exposed *in vitro* to the lysates exhibit marked proliferation and induce sarcoma formation when inoculated into nude mice. Additionally, the cells display increased duration of S phase, decreased apoptosis, down-regulation of the p27 tumor suppressor, and up-regulation of anti-apoptotic protein Bcl-2 [25,27,30,31]. Eggs of *S. haematobium* stimulate cellular proliferation, interfere with apoptosis, increase oxidative stress, and induce a genotoxicity on HCV-29 cells, derived from human urothelial cells [27,30–33]. In addition, 8-nitroguanine forms via inducible expression of nitric oxide synthase in Oct3/4-positive stem cells in UGS-associated bladder cancer tissue [34], and DNA nitrative and oxidative mutations characterized by 8-nitroguanine and 8-hydroxy-2′-deoxyguanosine (8-oxodG), have been implicated in the promotion of inflammation-mediated carcinogenesis by infection with *S. haematobium* [35].

Here we undertook analysis of urine from persons with urogenital schistosomiasis (UGS) living in endemic areas in Angola. Liquid chromatography diode array detection electron spray ionization mass spectrometry (LC-ESI-MS) revealed the presence of numerous estrogen metabolites in the urine of the study participants. Seven of these molecules were specifically identified in urine of the UGS cases but, notably, were not described in the recently reported metabolome of urine from healthy humans [36]. These metabolites were potentially reactive with host DNA: the molecules were either catechol estrogen quinone (CEQ) derivatives or CEQ-DNA-adducts. In addition, novel molecules derived from 8-oxodG during UGS were also identified.

Material and methods

Ethics statement

Informed consent was obtained directly from all participants or parents of underaged participants. Clinical-pathological information was obtained from clinical records of the participants. The Ethics Committee of Agostinho Neto University, Luanda, Angola, approved all procedures employed in this investigation.

Participants assigned to three categories

The participants were assigned to three groups: Group I included cases with urogenital schistosomiasis (UGS) and squamous cell carcinoma of the bladder (SCC); Group II included cases with UGS and with urothelial cell carcinoma (UCC), both histological types SCC and UCC (SSC+UCC) and papillary urothelial neoplasm of low malignant potential (PUNLMP); and Group III included cases with UGS but without cancer. Specifically, Groups I and II included discrete types of bladder cancer: Group I, seven participants with SCC; Group II included 11 cases of urothelial bladder carcinoma, essentially urothelial cell carcinoma (UCC) or mixed with SCC (Tables 1 and 2). Group III included 22 participants presenting non-neoplastic lesions such as chronic inflammation at various grades, including dysplasia, hyperplasia and metaplasia. Table 2 summarizes these details and Supplementary Table S1 provides clinical information and laboratory findings for all 40 participants.

Clinical cases, urine samples

Urine from 40 Angolans (67.5% females, 32.5% males), patients at the Sagrada Esperança Clinic and Hospital Américo Boavida, Luanda, was studied. Cases of urogenital schistosomiasis (UGS) were diagnosed when urine or tissue samples were positive for *S. haematobium* eggs by cystoscopy and/or biopsy. The median age of the UGS cases was 33.5 years, range 12 to 82 years, as described [37]. The study participants who donated the urine resided in the suburban regions of Luanda but most were originally from other Angolan provinces known to be endemic for infection with *S. haematobium* (http://www.who.int/schistosomiasis/epidemiology/en/angola_namibia.pdf). The detection of eggs of *S. haematobium* was undertaken by filtering 10 ml urine through a polycarbonate filter with a pore size of 12 μm in diameter (Millipore, UK). The slides were examined at ×100 under a compound microscope, according to guidelines provided by the WHO, http://www.who.int/mediacentre/factsheets/fs115/en/. One mg/ml final concentration of ascorbic acid was added [38–40] to a matched 10 ml sample, after which urine was stored at –20 °C, and freighted cold to Porto, Portugal for further analysis (below).

All the participants were examined by ultrasonography. Ultrasonographic studies of these participants with UGS revealed irregularities of the inner surface of the bladder wall and, in some cases, localized thickening of bladder wall protruding into the lumen was evident (not shown). The participants underwent cystoscopy and biopsy of the mass and corresponding adjacent mucosa. The apparently normal urothelium of cases without noticeable tumor mass were also subjected to random biopsy. All biopsies of apparently normal urothelium and tumor-adjacent mucosa presented benign/pre-malignant lesions, i.e. papilloma (P), chronic inflammation, urothelial hyperplasia, epidermoid metaplasia or dysplasia. Malignant lesions included papillary urothelial neoplasm of low malignant potential (PUNLMP), urothelial (= transitional) cell carcinoma (UCC), squamous cell carcinoma (SCC), and/or both UCC and SCC, as summarized in Table 1. Biopsy samples were examined and scored by co-author CL, with the pathological diagnosis based on the 2004 grading criteria of the WHO [41]. These criteria were used to organize the participants into the three groups summarized in Tables 1 and 2.

Table 1
Brief epidemiological parameters of study participants in Angola.

Parameter/characteristic	Number	Percentage
Number of participants	40	
UGS	40	100
UGS and associated cancers	18	45.0
UGS participants (Group I, II, III)	40	100
Age in years (mean)	33.5	
Range	12–82	
Gender		
Female	27	67.5
Male	13	42.5
Benign lesion		
Urothelial hyperplasia	12	30.0
Chronic inflammation	12	30.0
Epidermoid metaplasia	12	30.0
Dysplasia	4	10.0
Papilloma	1	2.0
Malignant lesion		
SCC	7	17.5
UCC	5	12.5
SCC + UCC	4	10.0
PUNLMP	2	5.0

UGS, urogenital schistosomiasis; SCC, squamous cell carcinoma; UCC, urothelial cell carcinoma (= transitional cell carcinoma); PUNLMP, papillary urothelial neoplasm of low grade malignant potential.

Liquid chromatography diode array detection electron spray ionization mass spectrometry

Samples were prepared and processed using liquid chromatography diode array detection electron spray ionization mass spectrometry, as described in References 27 and 30. In brief, methanol was added to the urine samples to 50% (volume/volume) and the samples stored at 4 °C until needed. Methanol displays acceptable

Table 2
Numbers and participant codes of samples of bladder tissue in the three study groups of persons with urogenital schistosomiasis (UGS) and with or without bladder cancer. The groups were assigned according to epidemiological and histological criteria. Code names were employed to de-identify the participants.

Group*	Disease status	Participant code name
I	SCC	B12, B20, B22, B27, B35, B37, B44
II	UCC	B2, B3, B6, B30, B42,
	UCC + SCC	B1, B5, B31, B38,
	PUNLMP	B14, B23
III	*Infection with S. haematobium*; with granuloma inflammation; Without bladder cancer (endemic or non-endemic)	B4, B8, B9, B10, B11, B13, B15, B16, B17, B18, B19, B21, B25, B26, B28, B29, B32, B33, B34, B36, B39, B43

SCC, squamous cell carcinoma; UCC, urothelial cell carcinoma (= transitional cell carcinoma), PUNLMP, papillary urothelial neoplasm of low malignant potential.
* All participants in the three groups, Groups I, II and III, were UGS positive.

chromatographic performance in terms of separation and sensitivity, with short gradient times [42]. High performance liquid chromatography (HPLC) coupled with mass spectrometer (MS) was employed to investigate relevant molecular species excreted in the urine. The MS analysis was performed within a LTQ Orbitrap XL mass spectrometer (Thermo Fischer Scientific, Bremen, Germany), fitted with an ultraviolet (UV) photo diode assay (PDA) detector. Analysis of samples involved a single injection of 20 µl of urine for chromatographic separation with a Macherey-Nagel Nucleosil C_{18}-column (250 mm × 4 mm i.d.; 5 µm particle diameter, end-capped). The mobile phase consisted of 1% formic acid in water (A)/acetonitrile (B) mixtures. Elution proceeded at a flow rate of 0.3 ml/min. Eluates were monitored for 75 min, run with a mobile phase gradient of 0–5 min, 100% A; 5–10 min, linear gradient from 100% to 80% A; 10–15 min 80% A; 15–50 min, linear gradient from 80 to 40% A; 50–65 min, 40% A; 65–75 min, linear gradient from 40% A to 100% B. Washing the column for 15 min with acetonitrile between each sample minimized carry-over, and also stabilized the column. Data were collected in negative electrospray ionization (ESI) negative mode scanning with an m/z range of 50 to 2000. The capillary voltage of the electrospray ionization was 28 kV, capillary temperature was 310 °C, flow rates of the sheath gas and auxiliary gas (N_2) were set to 40 and 10 (arbitrary units as provided by the software settings), respectively, and gas temperature was 275 °C. At the outset, the workflow was undertaken using three samples in order to confirm the analytical method and to stabilize the column. Thereafter, samples were analyzed in batches of six. Batch effects were not apparent.

Results

Urine estrogen-derived metabolites specifically related to squamous cell carcinoma of the bladder induced by urogenital schistosomiasis

Photo diode array (PDA) detector chromatograms of the UV spectra were obtained from urine. Mass spectral analysis was undertaken in negative mode, i.e. species were detected as [M-H] ions of the main constituents of urine from Groups I, II and III. The PDA chromatograms for each of the 40 urines are presented in Supplementary Fig. S1. Notable differences among the groups of participants were apparent. Seven specific peaks occurred consistently in urine of all participants with UGS (i.e. infected with *S. haematobium*): peaks at retention intervals of 22, 24, 30, 34, 36, 43 and 49 minutes. The m/z and molecules corresponding to these seven peaks are represented in Fig. 1. These peaks are highlighted in yellow (Group I, II) and orange (Group III) on representative PDAs from the three groups shown in Fig. 2.

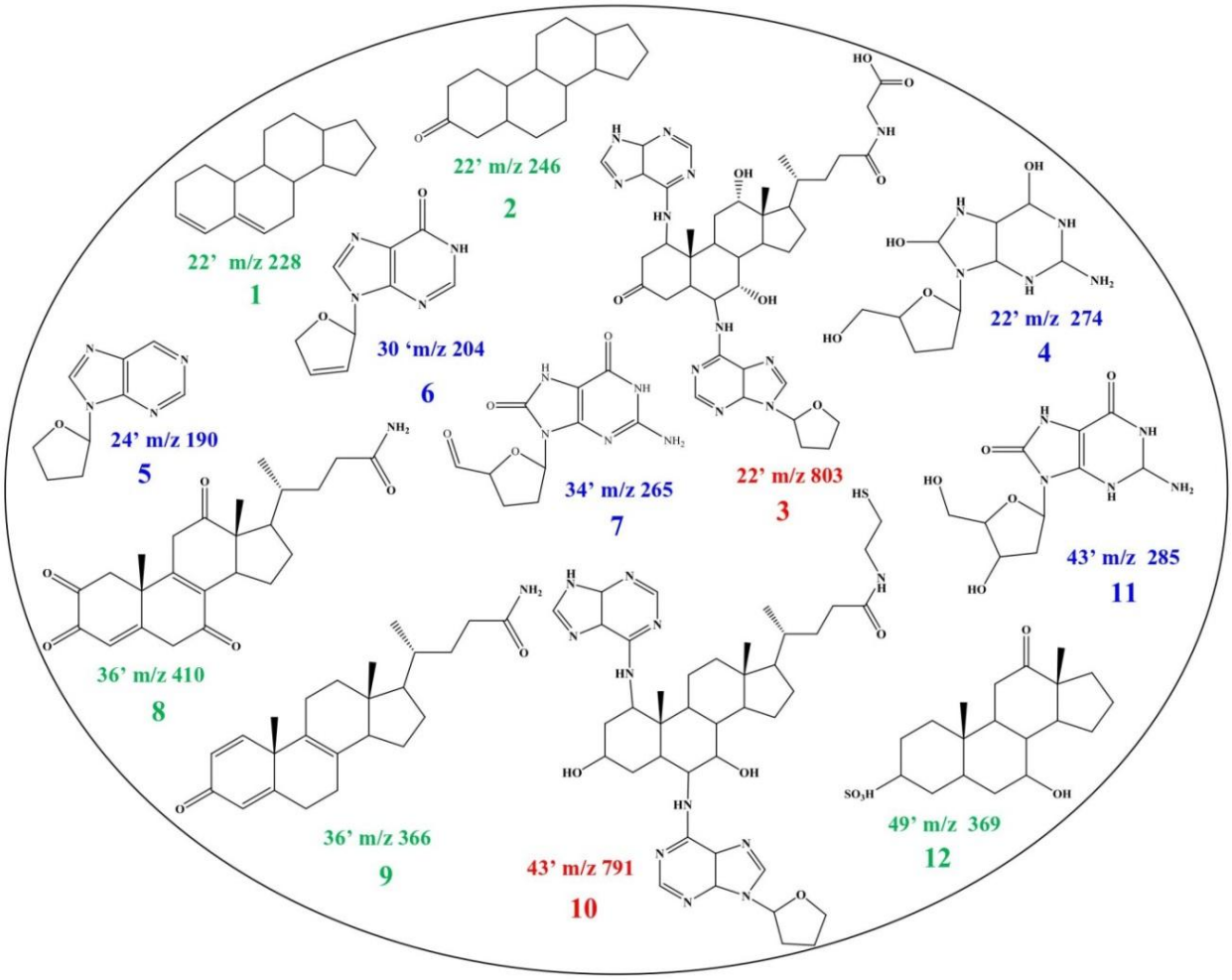

Fig. 1. Representative molecules identified in urine of individuals in Groups I, II and III, but which are not present in the database of the human urine metabolome. Comparing the m/z against a database of the human urine metabolome from healthy individuals [36], these molecules were not identified, indicative that they are related to *S. haematobium* infection. The retention time is the same for the three groups reported. The molecules highlighted by green colored font correspond to derivatives of catechol estrogen derivatives, red to catechol estrogen quinones-DNA adducts, and blue to derivatives of 8-oxodG.

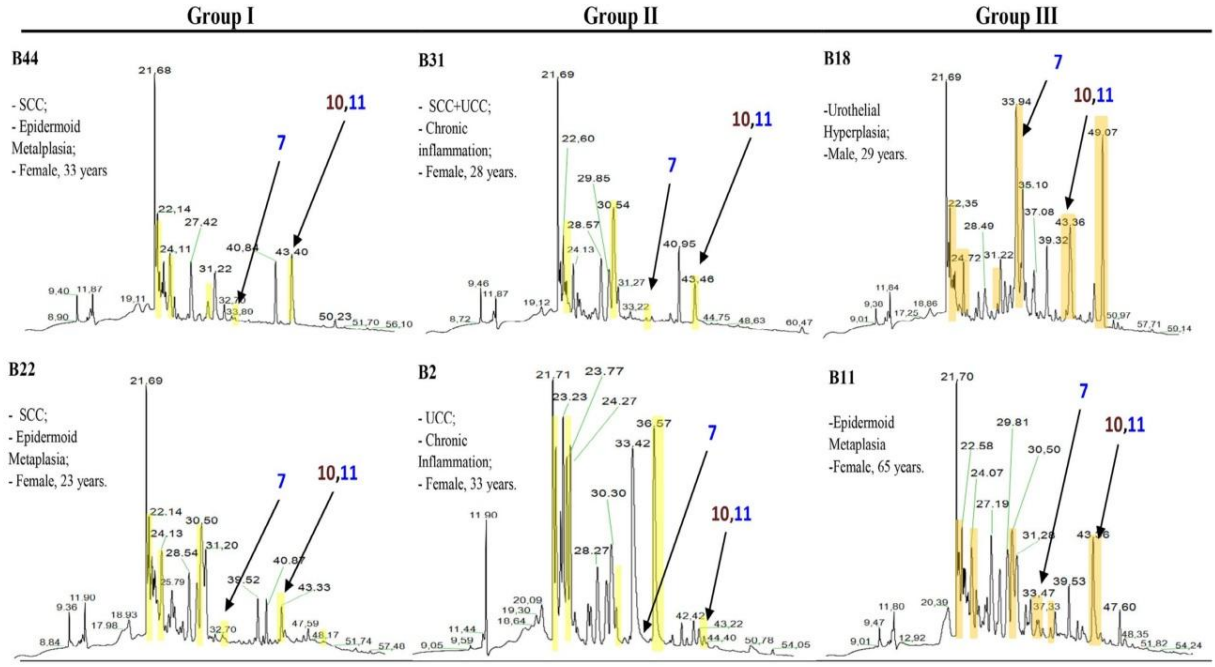

Fig. 2. PDA chromatograms of urine analyzed by LC-MS/MS, grouped according to histopathology of participants. The groups were organized according to the histopathology: Group I – patients with UGS and associated SCC cancer; Group II – patients with UGS and mixed urothelial cancer, and Group III – patients with UGS but free of cancer. Seven specific peaks were consistently seen in chromatograms from urine of all cases of *S. haematobium* infection (UGS): these are highlighted in yellow (Group I, II) and orange (Group III).

There were clear differences between Group III and Groups I/II, illustrated by fewer peaks in urine from participants with UGS-associated bladder cancer (Groups I/II). The peaks with retention times of 22, 24, 34, 36, 43, 49 minutes represented potential biomarkers for the presence of UGS (at some point in time) in individuals with bladder cancer. Further, the peaks at retention times of 34 and 43 minutes may be indicators that infection with S. *haematobium* continued in the persons during the appearance of carcinoma of the bladder. Curiously, the 8-oxodG derivatives related to these peaks, **7** and **11** were more pronounced in participants exhibiting UGS but without cancer, as well as estrogen-DNA-adduct **10** (Fig. 2). This might suggest that DNA oxidation processes are more pronounced in individuals close to the inception of carcinoma (Group III) compared to beyond the appearance of cancer (Group I/II). Once carcinoma in situ manifests, molecules **7**, **10** and **11** (Figs 1, 2) might be useful as urine biomarkers for detection and/or progression of bladder cancer.

The components associated with the indicated peaks were estrogen-like metabolites: derivatives of catechol-estrogen quinones (CEQ) (**1**, **2**, **8**, **9** and **12**) and estrogen quinone-DNA-adducts (CE-DNA-adducts) (**3**, **10**). Other relevant urine metabolites were directly related with 8-oxodG (**4**, **5**, **6**, **7** and **11**). The MS spectra, fragmentation patterns and corresponding retention times for these 12 compounds are presented in Supplementary Fig. S2. All peaks detected in the urine samples were included in the database presented as Supplementary Table S2. The CEQ derivatives are represented with m/z such as 228, 246, 366, 369, 410 and CEQ-DNA adducts present m/z values of 791 and 803 (Fig. 1). CEQ-DNA adducts **3** and **10** have been described previously in eggs and other developmental stages of *S. haematobium* [27,31]. By contrast, these molecules were not present in the catalog of metabolites (metabolome) of urine from healthy people [36].

Metabolites of 8-oxodG are associated with squamous cell carcinoma of the bladder induced by urogenital schistosomiasis

Of the seven peaks discussed above, and highlighted in Fig. 2, retention times of 22, 24, 30, 34, 36, 43 and 49 minutes, several appeared to be related to components that derive directly from 8-oxodG; these exhibited m/z 190, 204, 265, 274 and 285 (**5**, **6**, **7**, **4**, **11**) (Fig. 1). Since 8-oxodG is a representative marker for DNA oxidative damage during oxidative stress [34,43], the detection of 8-oxodG in urine samples herein may be clear evidence that DNA damage occurs in UGS. These reactive oxygen species induce oxidation, nitration, halogenations, and deamination of biomolecules, including nucleic acids, with the formation of toxic and mutagenic products [44]. These, in turn, can lead to DNA damage, eventually inducing mutations that have been implicated in the initiation and/or promotion of inflammation-mediated carcinogenesis [35]. Indeed, we postulate below that the 8-oxodG derivatives, **7** and **11** (retention intervals/peaks at 34 and 43 min**,** respectively; MS fragmentation profiles presented in Supplementary Fig. S3) represent putative biomarkers for diagnosis and/or prognosis of bladder cancer.

Discussion

Cancer of the urinary bladder is a major complication associated with chronic infection with *Schistosoma haematobium* in Africa and the Middle East [22,24,45,46]. Genetic alterations, chromosomal aberrations, and cytological changes have been described in carcinomas associated with UGS [22,24,47]. *N*-nitroso compounds are implicated as candidate etiologic agents in the process of bladder carcinogenesis [48]. Elevated levels of DNA alkylation damage in carcinomas associated with UGS and a high frequency of G to A transitions in the H-ras gene and in the CpG sequences of the p53 tumor suppressor gene also have been reported [22,47]. These outcomes indicate that UGS-associated SCC arises through a progressive accumulation of genetic changes in epithelial cells. Moreover, there is increasing evidence that endogenous DNA damage is a major etiological factor in human cancers [47].

Indicators of oxidative stress are readily detected in soluble lysates of the adult developmental stages of *S. haematobium* [27], and this mechanism is likely involved in induction of SCC during UGS [47]. Positive correlation between UGS and increase of levels of oxidative stress accompanied by continuous DNA damage and repair in urothelial carcinomas has been observed repeatedly [27,35,44]. In addition, the present findings revealed the presence in urine of molecules **3** and **10** (Fig. 1) known from adult worms and eggs of *S. haematobium* [27]. We have previously implicated a role of these reactive metabolites of schistosome origin in the carcinogenesis of UGS-associated SCC [27]. With respect to interactions of estrogen-related molecules from schistosomes on the endocrine and immune systems of the host, metabolites of estrogens can be considered as carcinogenic chemicals [49,50]. Hydroxylation of estrogens forms the 2- and 4-catechol estrogens involved in further oxidation to semiquinones and quinones, including the formation of the catechol estrogen-3, 4-quinones, the major carcinogenic metabolites of estrogen. These electrophilic compounds react with macromolecules including DNA to form the depurinating adducts that eventually lead to mutation and cancer. Several mechanisms explain the role of estrogen in disease. The better-known hypothesis is that estrogen receptor mediates cell proliferation, increasing errors in DNA replication [51,52]. Another interpretation postulates that estrogen metabolites react covalently with DNA bases by redox cycling or by forming a basic site. Subsequent error-prone repair of the modified DNA generates oncogenic mutations that initiate cancer. The two mechanisms may act in concert [53].

Likewise, metabolism of estrogens and the production of depurinating estrogen adducts can be postulated in a pathway underlying schistosomal-promoted damage to host genes. The carcinogenic effect of this estrogen-DNA adduct mediated pathway could partially explain the link between UGS and SCC of the bladder. UGS induced SCC is clearly multifactorial, however, in like fashion to other infection- and inflammation-related related cancers at large [23–27]. The chromatograms of urine of the UGS cases exhibited few metabolites with high mass range, which we speculate results from chemical processes, including hydrolysis occurring in the aqueous system. This might also explain the presence of more metabolites with higher mass, i.e. m/z 716, m/z 817 in schistosomes themselves. In any event, these findings supported the notion of a carcinogenic effect of an estrogen-DNA adducts mediated pathway in UGS-associated bladder cancer. Globally, the PDA chromatograms included in Groups I and II revealed fewer peaks compared with Group III. These findings are intriguing, and prompt several questions: could this situation reflect a decrease of the excreted metabolites; and might this phenomenon be predictive of cancer progression?

Comparing PDA chromatograms from individuals with UGS and with UGS-associated bladder cancer, there were common peaks that may indicate that the *S. haematobium* infection was responsible for the progression from infection-induced inflammation to bladder cancer. (The structural interpretation of MS data postulated for the novel molecules supported by MS fragmentation analysis is shown in Supplementary Figs S1 and S3.) Moreover, based on comparison of the m/z related to these peaks with a database of the human urine metabolome of healthy persons (from 22 persons in Canada) [36], we conclude that these novel molecules (Fig. 1) resulted from UGS. The peaks at 34 and 43 min retention time are probably indicators that infection with *S. haematobium* continued in the individuals with bladder cancer. The molecules related to peaks, **7**, **10** and **11**, might be useful as biomarkers to detect bladder cancer. These novel molecules proposed are derived directly from

8-hydroxy-2′-deoxyguanosine (8-oxodG), (**7** and **11**), as confirmed by MS fragmentation (Supplementary Fig. S3) or as adduct formation with estrogen (**10**).

8-oxodG is a major chromosomal lesion caused by oxidation, leading predominantly to G to T transversions [34,45–48,54,55]. The formation of 8-oxodG is regulated by local antioxidant capacity and DNA repair enzyme activity; 8-oxodG arises from the spontaneous oxidation of guanine, and is considered the major product of oxidation in DNA and the major source for mutations [33,37,43]. Marked elevation of 8-oxodG level squamous cell carcinoma-derived cells and bladder carcinomas associated with UGS when compared with non-schistosomal carcinomas has been described [47], suggestive of a strong correlation between UGS and increased levels of oxidative stress accompanied by a continuous DNA damage and repair in carcinomas of the urinary bladder [47]. 8-Nitroguanine is produced in the neoplasms of *S. haematobium*-associated bladder cancer patients [35]. Formation of 8-nitroguanine and 8-oxodG was significantly higher in bladder cancer and cystitis tissues than in healthy tissues [35]. 8-oxo-dG is a pivotal marker for measuring the effect of endogenous oxidative damage to DNA and has a factor of initiation and promotion of carcinogenesis [56,57]. Here we confirmed the presence of 8-oxodG in the urine of persons with UGS with or without bladder cancer, which supports the notion that metabolites of estrogen originating from the schistosome induce lesions in host cell chromosomes [31,41].

Whereas mass spectroscopy in isolation cannot assign structure conclusively, and moreover, we have been cautious in over-interpreting the LC-MS data, here we have determined structures that were consistent with the literature [39,49] and with our previous findings related to UGS and tissues and secretions of the parasite [25,27,30–33,37]. Furthermore, the structural interpretation of mass spectrometric data for the novel molecules was supported by MS fragmentations (Supplementary Figs S2, S3). In overview, these metabolites were excreted in urine and, based on molecular structure they may originate in the schistosome. We have previously reported metabolites **3** and **10** in *S. haematobium* eggs and adult worms [27]. Each of the metabolites shown in Fig. 1 potentially can react with DNA to induce apurinic sites, leading to DNA lesions and mutations that, in turn, can initiate carcinogenesis [39,49]. Biomarkers would be informative in early detection and prognosis of malignancy induced by UGS. Promising candidates, notably (1) estrogen-like and (2) 8-oxodG related metabolites highlighted here, appear worthy of validation in larger population based studies. Also, we caution that the malignant lesions included here included both UCC and SCC. Mixed urothelial cancer with squamous features, such as some cases in Group II, may have distinct biology compared to UGS related 'pure' SCC, and moreover the literature clearly indicates that UGS is a risk factor for SCC but not UCC [22–29]. Accordingly, investigation of larger populations may also facilitate the characterization of metabolites that characterize the discrete pathogenesis of SCC and UCC. These and related questions as noted above might also be addressed using informative cell lines, urine from UGS/SCC cases, and/or purified metabolites. Moreover, the novelty of metabolites excreted in urine during UGS suggests basic mechanisms for carcinogenesis in UGS-induced SCC, and maybe in other infection-related malignancies.

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Conflict of interest

The authors declare no conflicts of interest in preparing this article.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2015.01.018.

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Capítulo 5

P53 and Cancer-Associated Sialylated Glycans Are Surrogate Markers of Cancerization of the Bladder Associated with Schistosoma haematobium Infection, in *PLOS Neglected Tropical Diseases*.

CAPÍTULO 5

P53 and Cancer-Associated Sialylated Glycans Are Surrogate Markers of Cancerization of the Bladder Associated with *Schistosoma haematobium* Infection, *in PLOS Neglected Tropical Diseases*.

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O artigo apresentado como Capítulo 5 tinha um propósito: conhecer e identificar alguns biomarcadores de cancro de bexiga em biópsias de pacientes com UGS. O artigo é explícito na descrição dos materiais e métodos utilizados bem como na identificação dos biomarcadores selecionados: p53, Ki-67 (proliferação dos tumores), dois glicanos da superfície celular associado ao cancro (sLea / sLex), envolvidos na evasão imunitária e metástase. Este estudo preliminar sugere que a p53 e glicanos sialilados são biomarcadores de cancerização da bexiga associado à UGS. Enfatizam os resultados a existência de um elo entre o parasita e as células tumorais: os ovos de *S. haematobium* expressam os antigénios sLea e sLex, mimetizando os leucócitos humanos; tal facto pode desempenhar um papel na colonização e disseminação do tumor. No entanto, e para efeitos dos nossos propósitos no presente ato doutoral, uma evidência atraiu a nossa atenção: a p53 está alterada num número muito significativo dos doentes com UGS, incluído no grupo ainda sem cancro.

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P53 and Cancer-Associated Sialylated Glycans Are Surrogate Markers of Cancerization of the Bladder Associated with *Schistosoma haematobium* Infection

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Abstract

Background: Bladder cancer is a significant health problem in rural areas of Africa and the Middle East where *Schistosoma haematobium* is prevalent, supporting an association between malignant transformation and infection by this blood fluke. Nevertheless, the molecular mechanisms linking these events are poorly understood. Bladder cancers in infected populations are generally diagnosed at a late stage since there is a lack of non-invasive diagnostic tools, hence enforcing the need for early carcinogenesis markers.

Methodology/Principal Findings: Forty-three formalin-fixed paraffin-embedded bladder biopsies of *S. haematobium*-infected patients, consisting of bladder tumours, tumour adjacent mucosa and pre-malignant/malignant urothelial lesions, were screened for bladder cancer biomarkers. These included the oncoprotein p53, the tumour proliferation rate (Ki-67> 17%), cell-surface cancer-associated glycan sialyl-Tn (sTn) and sialyl-Lewis$^{a/x}$ (sLe^a/sLe^x), involved in immune escape and metastasis. Bladder tumours of non-*S. haematobium* etiology and normal urothelium were used as controls. *S. haematobium*-associated benign/pre-malignant lesions present alterations in p53 and sLe^x that were also found in bladder tumors. Similar results were observed in non-*S. haematobium* associated tumours, irrespectively of their histological nature, denoting some common molecular pathways. In addition, most benign/pre-malignant lesions also expressed sLe^a. However, proliferative phenotypes were more prevalent in lesions adjacent to bladder tumors while sLe^a was characteristic of sole benign/pre-malignant lesions, suggesting it may be a biomarker of early carcionogenesis associated with the parasite. A correlation was observed between the frequency of the biomarkers in the tumor and adjacent mucosa, with the exception of Ki-67. Most *S. haematobium* eggs embedded in the urothelium were also positive for sLe^a and sLe^x. Reinforcing the pathologic nature of the studied biomarkers, none was observed in the healthy urothelium.

Conclusion/Significance: This preliminary study suggests that p53 and sialylated glycans are surrogate biomarkers of bladder cancerization associated with *S. haematobium*, highlighting a missing link between infection and cancer development. Eggs of *S. haematobium* express sLe^a and sLe^x antigens in mimicry of human leukocytes glycosylation, which may play a role in the colonization and disease dissemination. These observations may help the early identification of infected patients at a higher risk of developing bladder cancer and guide the future development of non-invasive diagnostic tests.

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Author Summary

Epidemiological studies associate infection with *S. haematobium*, an endemic parasitic flatworm in Africa and the Middle East, with the development of bladder cancer. Nevertheless, little molecular evidence exists supporting this association. This work draws attention to the common molecular pathways underlying these two events, highlighting a potentially unreported link between infection and cancer development. It has been demonstrated that a panel of biomarkers commonly associated with aggressive forms of bladder cancer is also present in non-malignant tissues infected with the parasite. This may offer a means of early identification of people with this parasitic infection who are at risk of developing of bladder cancer, and may guide the establishment of non-invasive diagnostic tests. Furthermore, we observed that parasite eggs mimic the molecular nature of human cells, providing a possible mechanism of immune escape and persistent infection. Such knowledge is considered pivotal to develop novel therapeutic strategies.

Introduction

Schistosoma haematobium, a parasitic flatworm infecting millions of people in Angola and other countries of Africa and Middle East, is responsible for the development of urinary schistosomiasis, a neglected tropical disease [1,2]. The World Health Organization estimates that 500 to 600 million people residing in rural agricultural and periurban areas are at risk of infection and over 200 million people are currently infected, 10% of which will experience sever health complications; [3,4].

The parasite has a complex life cycle consisting of two phases, one inside the human body (the definitive host) and another inside a snail of the genus *Bulinus* [5]. Free-swimming cercariae penetrate human skin when in contact with contaminated water, enter the blood stream and travel to the liver to mature into adult flukes. After a period of about three weeks the young flukes migrate to the plexuses around the urinary bladder to copulate. The eggs released by female flukes traverse the wall of the bladder causing haematuria, fibrosis and ultimately the calcification of the tissue; they are then excreted through urine [6,7]. However, some eggs become embedded in the bladder mucosa further contributing to chronic inflammation and granuloma formation [6,7]. The eruption of the eggs through the mucosa stimulates not only the establishment of chronic inflammations but also promotes the development of benign/pre-malignant bladder lesions such as urothelial hyperplasia and dysplasia that may be precursors of bladder cancer [8–10]. When contaminated urine comes in contact with fresh watercourses (e.g. rivers), the eggs hatch, releasing free-swimming miracidia that infect the intermediate snail host. After a maturation period new cercariae are formed and released into the environment, assuring the perpetuation of infection and transmission of the disease [5].

The World Health Organization (WHO) International Agency for Cancer classifies *S. haematobium* as a Group 1 biological carcinogen, a definitive cause of cancer [11]. Epidemiological findings reveal a positive relationship between *S. haematobium* infection and the development of squamous cell carcinoma of the bladder, a type of bladder cancer rarely observed in western patients but prevalent in Africa and Middle East [12–14]. It has been observed that patients infected with the parasite have a higher risk of developing bladder cancer earlier in life than uninfected people [13,15]. The probability of developing cancer has been suggested to depend on the intensity (worm burden and tissue egg burden) and duration of infection [16,17]. However, despite the epidemiological data from case control studies and the geographical overlap between bladder cancer development and regions endemic for urogenital schistosomiasis [8,18], few experimental evidences support this association. Nonetheless, Botelho and coworkers demonstrated recently that the exposure to soluble antigen extracts of mixed sex adult *S. haematobium* worms and eggs promote the tumourogenic potential of urothelial cells in *vitro* and *in vivo* [8,19,20], and Zhong and colleagues have reported hypermethylation of several genes including *RASSF1A* and *TIMP3* detected in urine sediments of Ghanaians with bladder pathology associated with infection with *S. haematobium* [21].Further understanding of the pathobiological features underlying the association between *S. haematobium* and bladder cancer development are needed to support these observations. The identification of the molecular events underlying early urothelial carcinogenesis in the bladder is also warranted. This is a particular critical matter since early symptoms of schistosomiasis, which include urinary pain and hematuria, are common to those of bladder cancer. As such, they are often neglected by local communities in developing countries, where medical assistance is scarce. Therefore, bladder tumours are often diagnosed at a late stage, which is associated with decreased overall survival. The identification of biomarkers may help to control *S. haematobium*-associated bladder cancer in these populations.

This research is based on establishing common molecular alterations among schistosoma-associated tumours and benign/pre-malignant lesions found either in tumor-adjacent mucosa or in apparently normal urothelia of cases without tumors. These lesions were screened for oncoprotein p53 that is associated with both aggressive urothelial [22–24] and squamous cell bladder carcinomas [25]. The proliferation rate, given by the overexpression of nuclear protein Ki-67, and considered a prognostic marker of tumor recurrence and progression in non-muscle invasive urothelial carcinoma [26–28], was also evaluated. Particular attention was further devoted to the characterization of alterations in membrane-bound glycans that accompanied malignant transformations and favor cell-to-cell detachment, migration, immune evasion and metastization [29]. This includes the sialylated antigens sialyl-Tn (sTn; CA72-4) [30,31], sialyl-Le^a (sLe^a, CA19-9) [32–34] and sialyl-Le^x (sLe^x) [35,36] that have been observed in bladder cancer. Cancer-associated glycans can also be found in secreted proteins often shed into the bloodstream and urine, offering potential for non-invasive diagnosis [34,36–38].

Materials and Methods

Ethics Statement

All procedures were performed after patient's written informed consent and parental consent in the cases of children and approved by the Ethics Committee of Agostinho Neto University, Luanda, Angola and the Portuguese Institute For Oncology of Porto, Portugal (IPO-Porto). Clinico-pathological information was obtained from patients' clinical records and this information was anonymized.

Cases

This study includes 43 Angolan patients (30.2% male and 69.8% female) diagnosed as positive for *S. haematobium* infection in Sagrada Esperança Clinic (Luanda, Angola) and Hospital Américo Boavida (Luanda, Angola). The median age of the patients was 33.5 years (12–82 years) and, even though the majority resided in the rural areas around Luanda, they were born and had resided in provinces where *S. haematobium* is endemic.

Table 1. Pathological characterization of the samples.

Benign/Pre-malignant lesions	No tumour (n = 24)	With Tumour (n = 19)				
		P	PTLMP	UC	SCC	UC+SCC
Chronic Inflammation	6	1	-	4	-	1
Urothelial Hyperplasia	8	-	2	-	2	1
Epidermoid Metaplasia	7	-	-	-	5	2
Displasia	3	-	-	-	-	-

P: papilloma; PTLMP: papillary tumour of low malignant potential; UC: urothelial tumour; SCC: squamous cell carcinomas; UC+SCC: urothelial cancer and squamous cell carcinoma.

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All patients presented irregularities of inner surface of bladder wall found by ultrasound scan and some of them showed a localized thickening of bladder wall protruding into the lumen. Therefore, the patients underwent cystoscopy and biopsy of the visualized mass and corresponding adjacent mucosa. The apparently normal urothelium of cases without noticeable tumour mass were also subjected to random biopsies. All biopsies of apparently normal urothelium and tumour-adjacent mucosa presented benign/pre-malignant lesions (chronic inflammation, urothelial hyperplasia, epidermoid metaplasia or dysplasia). Malignant lesions included papilloma (P), papillary urothelial neoplasm of low malignant potential (PUNLMP) and high-grade urothelial cell carcinoma (UCC), squamous cell carcinomas (SCC) or both (UCC+SCC) as summarized in Table 1. No differences were observed in age and sex distribution among the lesions/tumours. *S. haematobium* eggs were evident in the bladder of 27 (62.8%) cases, from these 7 (26%) presented tumours.

This study also includes a retrospective series of 22 non-*Schistosoma haematobium* infected patients diagnosed with urothelial cell carcinoma (10 low-grade tumours; 12 high-grade tumours, 5 presenting muscle invasion) and 4 squamous cell carcinomas presenting invasion of the *muscularis propria*, that have been previously characterized in relation to Ki-67 and sTn expressions by Ferreira *et al.* [31]. The patients (48.3% male and 51.7% female), mean age 69 years (45-89 years), underwent transurethral resection of the tumour in the Portuguese Institute for Oncology of Porto (IPO-Porto, Portugal), between July 2011 and May 2012. None had received prior adjuvant therapy. Six normal urothelium tissues of necropsied male individuals without bladder cancer history, within the same mean of age range, were also included.

Formalin fixed paraffin embedded biopsies and tumour sections stained with hematoxylin and eosin were examined and classified by an experienced pathologist under light microscopy, with reference to the WHO's 2004 grading criteria [39].

Immunohistochemistry

Formalin-fixed, paraffin-embedded (FFPE) tissue sections were screened for p53 accumulation, proliferation (Ki-67), and cancer-associated glycans sTn, sLe^a, and sLe^x by immunohistochemistry by the streptavidin/biotin peroxidase method using mouse monoclonal antibodies. The p53 protein was determined with clone DO-7 (Dako), Ki-67 with clone MIB-1 (Dako), sTn with clone TKH2 [31], sLe^a with clone (Abcam) and sLe^x with clone (Abcam). Briefly, 3 µm sections were deparaffinized with xylene, rehydrated with graded ethanol series, microwaved for 15 min in boiling citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0), and exposed to 3% hydrogen peroxide in methanol for 20 min. After blockage with BSA (5% in PBS), the antigens were identified with UltraVision Detection System (Thermo Scientific) followed by incubation with 3,3-diaminobenzidine tetrahydrochloride (Impact Dab, Vector). Finally, the slides were counterstained with haematoxylin for 1 min. Colon carcinoma, tonsil and intestinal metaplasia tissue sections were tested in parallel as positive controls for, p53, Ki-67 and sialylated glycans, respectively. Negative control sections were included, involving sections probed with BSA (5% in PBS) devoid of primary antibody. The tissues were also treated with a neuraminidase from *Clostridium perfringens* (Sigma-Aldrich) to remove the sialic acid from the glycans and screened thereafter for sTn, sLe^a, and sLe^x, as described by Ferreira et al. [31].

A semi-quantitative approach was established to score the immunohistochemical labeling based on the intensity of staining and the percentage of cells that stained positively. The immunoexpression was assessed blindly by two independent observers and validated by an experienced pathologist. Whenever there was a disagreement, the slides were reviewed, and consensus was reached. Tumours were classified as p53 positive whenever expression was higher than 5% of the tissue section, as proliferative whenever Ki-67 expression was higher than 17%, as described by Santos et al. [26], and sTn, sLe^a and sLe^x were considered positive whenever the percentage of staining was $\geq$5% of the tissue sections [31,32,35].

Statistics

Statistical data analysis was performed using the IBM Statistical Package for Social Sciences—SPSS for Windows (version 20.0). Chi-square analysis was used to compare categorical variables. Correlation between cancer associated markers expression in pre-malignant lesions and concomitant tumours whenever present was performed using Pearson correlation test. A P value of $\leq$0.05 was considered to be statistically significant.

Results

Bladder tumours associated with *S. haematobium* infection were screened for the accumulation of p53, proliferation rate (Ki-67> 17%) and cancer-associated sialylated glycans sTn, sLe^a and sLe^x (Fig. 1). We could observe that all the biomarkers were expressed throughout the different layers of the urothelium in benign/pre-malignant lesions and also homogeneously expressed in the tumours, irrespectively of their histological classification. As presented in Table 2, the majority of the bladder tumors exhibited p53 alterations (84%) and sLe^x overexpression (74%). Similar percentages of p53 and sLe^x could also be observed in bladder tumour sections from patients non-infected with *Schistosoma haematobium*, irrespectively of their histological natures. Conversely, non-proliferative phenotypes predominated among low malignant lesions (papilloma and PUNLMP; 100% of the cases)

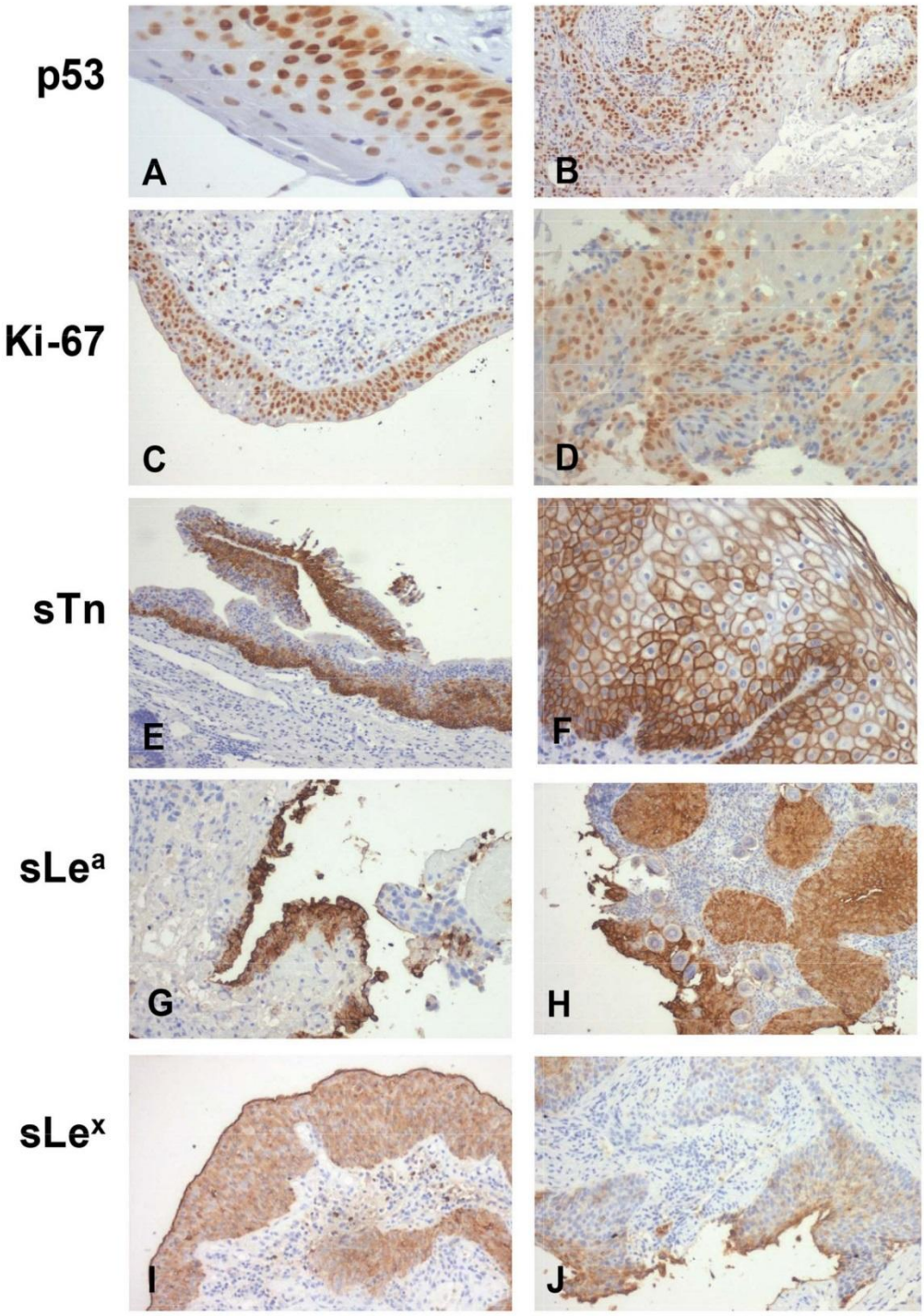

Fig. 1. Expression of cancer-associated biomarkers p53, Ki-67, sTn, sLe^a and sLe^x in bladder benign/pre-malignant lesions. A) Chronic inflammation; B) SCC; C) Chronic inflammation; D) SCC; E) Urothelial hyperplasia; F) Urothelial carcinoma; G) Dysplasia; H) SCC; I) Dysplasia; J) Urothelial carcinoma.
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Table 2. Cancer associated markers expression in *Schistosoma haematobium*-associated bladder tumours.

Variables	Papilloma	PUNLMP	UCC	SCC	UCC+SCC	Total
	n (%)	n (%)	n (%)	n(%)	n(%)	n (%)
p53						
Negative	0 (0.0)	0 (0.0)	0 (0.0)	2 (28.6)	1 (25.0)	3 (15.8)
Positive (altered)	1 (100.0)	2 (100.0)	5 (100.0)	5 (71.4)	3 (75.0)	16 (84.2)
Ki-67						
Non proliferative	1 (100.0)	2 (100.0)	2 (40.0)	3 (42.9)	2 (50.0)	10 (52.6)
Proliferative (>17%)	0 (0.0)	0 (0.0)	3 (60.0)	4 (57.1)	2 (50.0)	9 (47.4)
sTn						
Negative	1 (100.0)	1 (50.0)	5 (100.0)	4 (57.1)	1 (25.0)	12 (63.2)
Positive	0 (0.0)	1 (50.0)	0 (0.0)	3 (42.9)	3 (75.0)	7 (36.8)
sLe^a						
Negative	-	1 (50.0)	2 (40.0)	4 (57.1)	2 (50.0)	10 (52.6)
Positive	-	1 (50.0)	3 (60.0)	3 (42.9)	2 (50.0)	9 (47.4)
sLe^x						
Negative	0 (0.0)	0 (0.0)	1 (20.0)	1 (16.7)	2 (50.0)	4 (22.2)
Positive	1 (100.0)	2 (100.0)	4 (80.0)	5 (83.3)	2 (50.0)	14 (77.8)

PUNLMP: Papillary Urothelial Neoplasm of Low Malignant Potential.
UCC: Urothelial Cell Carcinoma.
SCC: Squamous Cell Carcinoma.
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when compared to the other groups comprehending more aggressive lesions presenting either invasion and/or high potential to invade the bladder wall (UCC, SCC, SCC+UC; approximately 50% of the cases) (Table 2). Contrasting with these findings, the percentage of proliferative phenotypes in the less aggressive non-schistosome associated lesions (low grade papillary tumours) was 30%. The percentage of proliferative phenotypes in more aggressive high grade lesions, including high grade urothelial cell and squamous cell carcinomas, was similar to described for *S. haematobium* infection related lesions. The sLe^a antigen was detected in approximately 50% of the *S. haematobium*-associated malignant lesions, irrespectively of their histology. These observations contrasted with the significantly higher expression of sLe^a observed in lesions not associated with the parasite (80% of the cases). Regarding the sTn antigen, its frequency varies among the histological groups of tumours, papilloma and UCC did not express the antigen, PUNLMP and SCC showed an equal distribution of negative and positive cases. However, the antigen was expressed by 75% of the cases presenting both an UCC and SCC phenotype, which is in accordance with our previous results for non-schistosome associated bladder tumors, where sTn antigen was present in approximately 75% of aggressive bladder tumors (high grade papillary tumors and muscle invasive bladder cancer) [31]. Altogether, the studied biomarkers, with the exception of sLe^a presented similar expressions in both schistosome and non-schistosome associated bladder tumors.

Benign/pre-malignant lesions associated with *S. haematobium* infection, irrespective of having been isolated from urothelium without malignant lesions or tumour adjacent mucosa, were also studied (Fig. 1). The p53 protein was altered in the majority of the benign/pre-malignant lesions (88%), predominantly in those showing cellular alterations (urothelial hyperplasia, epidermoid metaplasia, dysplasia; Table 3). Approximately 40% of the cases also presented a high proliferation index (Ki-67>17%), although this was more pronounced among epidermoid metaplasia (64.3%, Table 3). The sTn antigen was detected in one third of the lesions (32.6%), however it was mainly absent in chronic inflammation cases and when compared with all the others a trend association was observed ($P = 0.067$). The sialylated Lewis antigens sLe^a and sLe^x were detected in the majority of the cases (>80%). However, the percentage of sLe^a positive cases was higher among the cases with urothelial hyperplasia while sLe^x was present in all dysplasia cases (4/4; Table 3). Furthermore, none of the normal urothelium tissues were positive for the studied biomarkers, demonstrating its cancer-associated nature.

Table 4 further highlights the relationship between the studied markers in the benign/pre-malignant lesions identified in apparently normal bladder mucosa and those found in tumor adjacent mucosa. The distribution of the p53 alterations, sTn and sLe^x antigens overexpression was similar between the two groups. However, a higher number of non-proliferative cases were observed in sole benign/pre-malignant lesions when compared with the lesions in tumour adjacent mucosa (73.9% vs 42.1%, $P = 0.037$; Table 4). On the other hand, sLe^a expression was more frequent in lesions without tumour than with concomitant tumours (91.7% vs 66.7%; $P = 0.03$). Altogether this data shows that the majority of the benign/pre-malignant lesions associated with *S. haematobium* infection share alterations in p53 expression and sLe^x with bladder tumors. The predominance of sLe^a in pre-malignant lesions, in particular in bladders that do not present signs of malignant transformation, suggesting that this glycan may be a molecular alteration associated with early carcinogenesis pathways.

Table 5 further shows the correlation between the expression of the biomarkers in the tumours and adjacent mucosa lesions. This showed a correlation between the expression of p53, sTn, sLe^a and sLe^x both in the lesion and tumour, denoting that the tumour adjacent mucosa reflects the molecular alterations found in S. *haematobium*-associated tumours.

Table 3. Cancer associated markers expression in benign/pre-malignant lesions.

Variables	Chronic Inflammation	Urothelial Hyperplasia	Epidermoid Metaplasia	Dysplasia	Total
	n (%)	n (%)	n (%)	n(%)	n (%)
p53					
Negative	3 (25.0)	1 (7.7)	1 (7.7)	0 (0.0)	5 (11.9)
Positive (altered)	9 (75.0)	12 (92.3)	12 (92.3)	4 (100.0)	37 (88.1)
Ki-67					
Non proliferative	6 (54.5)	11 (84.6)	5 (35.7)	3 (75.0)	25 (59.5)
Proliferative (>17%)	5 (45.5)	2 (15.4)	9 (64.3)	1 (25.0)	17 (40.5)
sTn					
Negative	11(91.7)	8 (61.5)	8 (57.1)	2 (50.0)	29 (67.4)
Positive	1 (8.3)	5 (38.5)	6 (42.9)	2 (50.0)	14 (32.6)
sLe^a					
Negative	4 (33.3)	1 (7.7)	3 (21.4)	1 (25.0)	8 (19.0)
Positive	8 (67.7)	12 (92.3)	11 (78.6)	3 (75.0)	34 (81.0)
sLe^x					
Negative	3 (25.0)	2 (15.4)	3 (21.4)	0 (0.0)	8 (18.6)
Positive	12 (75.0)	11 (84.6)	11 (78.6)	4 (100.0)	35 (81.4)

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Moreover, it was observed that 60% of the cases presented *S. haematobium* eggs embedded in the bladder urothelium, predominantly in benign/pre-malignant lesions without the presence of tumour (75% vs 45%). However, no associations were found between the expression of the studied markers and the presence and absence of the eggs in the bladder at the time of diagnosis. Altogether, these findings suggest that the presence of eggs in the bladder may be an early event leading to carcinogenesis. Whether the disorganization of the tissue associated with malignant transformation may favor their release into the environment, therefore explaining is lower presence in malignant tissues and consequently the lack of correlation with the studied biomarkers, warrants further investigation. It was further observed that the majority of the cases (>75%) presented sLe^a and sLe^a positive eggs

Table 4. Relation between cancer associated markers in benign/pre-malignant lesions as sole lesions and in tumour adjacent mucosa.

Variables	Benign/Pre-malignant		
	As sole lesion	In tumour adjacent mucosa	p*
	n (%)	n (%)	
p53			
Negative	1 (4.2)	4 (22.2)	
Positive (altered)	23 (95.8)	14 (77.8)	0.146
Ki-67			
Non proliferative	17 (73.9)	8 (42.1)	
Proliferative (>17%)	6 (26.1)	11 (57.9)	0.037
sTn			
Negative	15 (62.5)	14 (73.7)	
Positive	9 (37.5)	5 (26.3)	0.437
sLe^a			
Negative	2 (8.3)	7 (36.8)	
Positive	22 (91.7)	12 (63.2)	0.030
sLe^x			
Negative	4 (16.7)	4 (21.1)	
Positive	20 (83.3)	15 (78.9)	0.714

*: Chi-square test;

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Table 5. Correlation between cancer associated marker expression in the lesions and in the concomitant tumour.

	Bladder Cancer									
Benign/Pre-malignant Lesions	p53		Ki-67		sTn		sLe^a		sLe^x	
	Correlation coefficient	*P* value	Correlation Coefficient	*P* value	Correlation Coefficient	*P* value	Correlation Coefficient	*P* value	Correlation Coefficient	*P* value
p53	0,484	**0,036**	–0,060	0,814	0,033	0,896	0,033	0,901	0,346	0,174
Ki-67	–0,057	0,811	0,382	0,106	0,025	0,918	–0,426	0,078	–0,570	**0,014**
sTn	–0,031	0,898	–0,378	0,171	0,457	**0,049**	0,372	0,128	0,286	0,250
sLe^a	0,016	0,945	–0,236	0,346	0,175	0,486	0,471	**0,048**	0,171	0,512
sLe^x	0,140	0,556	–0,027	0,912	0,015	0,950	0,000	1,000	0,679	**0,002**

doi:10.1371/journal.pntd.0003329.t005

and approximately half displayed eggs expressing the sTn antigen, suggesting some degree of mimicry of host glycosylation patterns (Fig. 2). The expression of sialylated glycans was validated by observing the loss of reactivity against anti-glycans monoclonal antibodies after treatment of the tissue with a neuraminidase. It was noteworthy that both positive and negative eggs for these antigens could be found within the same biopsy, denoting some degree of heterogeneity at this level.

Discussion

In contrast to the extensive cytogenetic and molecular signatures existing for urothelial cell carcinoma, mainly found in western populations, little is known about the molecular alterations underlying the development of *S. haematobium*-associated bladder cancer. Nevertheless, such information is pivotal to support a definitive association between schistosomiasis and bladder cancer development that, until now, has been mostly supported by epidemiological studies. Furthermore, it may provide means for an early identification of infected populations at a risk of developing bladder cancer, which is a particular critical matter since the majority of the cases are detected at a late stage due the absence of appropriate medical facilities.

Herein we have screened a series of schistosome-associated bladder tumours, their adjacent mucosa and also biopsies of apparently normal urothelia, for bladder cancer biomarkers. We also included a series of bladder tumours of non-schistosome etiology and normal urothelium sections in an attempt to highlight common molecular alterations. The majority of the patients enrolled in this study were females, and this is in clear contrast with the higher prevalence of bladder cancer among men in western populations. This may be explained by social and working habits of these populations living in the proximity of contaminated water courses. Also, the majority of the cases of bladder cancer were observed among young adults, which is rare for urothelial carcinomas of chemical etiology [40]. These findings were in accordance with previous observations from other authors [5,8] and support a role for the parasite in cancer development.

The evaluated biomarkers included the accumulation of oncoprotein p53 [22–24] and tumour proliferation index given by Ki-67 overexpression [26–28], two events associated with the aggressiveness of urothelial bladder cancer. Likewise, we observed alterations in p53 in the majority of schistosome-associated bladder tumours, irrespectively of their histopathological nature, which is in agreement with our findings and previous observations for non-schistosome associated tumours [25,41]. In addition, these alterations were not observable in the normal urothelium, denoting its cancer-related nature. The association between the accumulation of p53 in the urothelium and infection with *S. haematobium* reinforces the notion that the parasite may contribute to profound alterations in urothelial cells, ultimately leading to aggressive forms of cancer. This hypothesis is further supported by the observations reported by Botelho and colleagues [8,19]. According to these authors, the exposure to *S. haematobium* antigens down-regulates cell apoptotic pathways, which would ultimately lead to the development of cancer [42]. Regarding proliferation, Ki-67 overexpression was lower in low malignant potential lesions when compared to urothelial and squamous cell carcinomas. This is in accordance with previous findings associating the degree of severity of bladder malignant lesions with higher proliferation degrees and potential to evolve to more aggressive forms of cancer [26,31]. Since several markers were evaluated and a large numbers of comparisons were performed, the false discovery rate should be considered. Nevertheless, if all

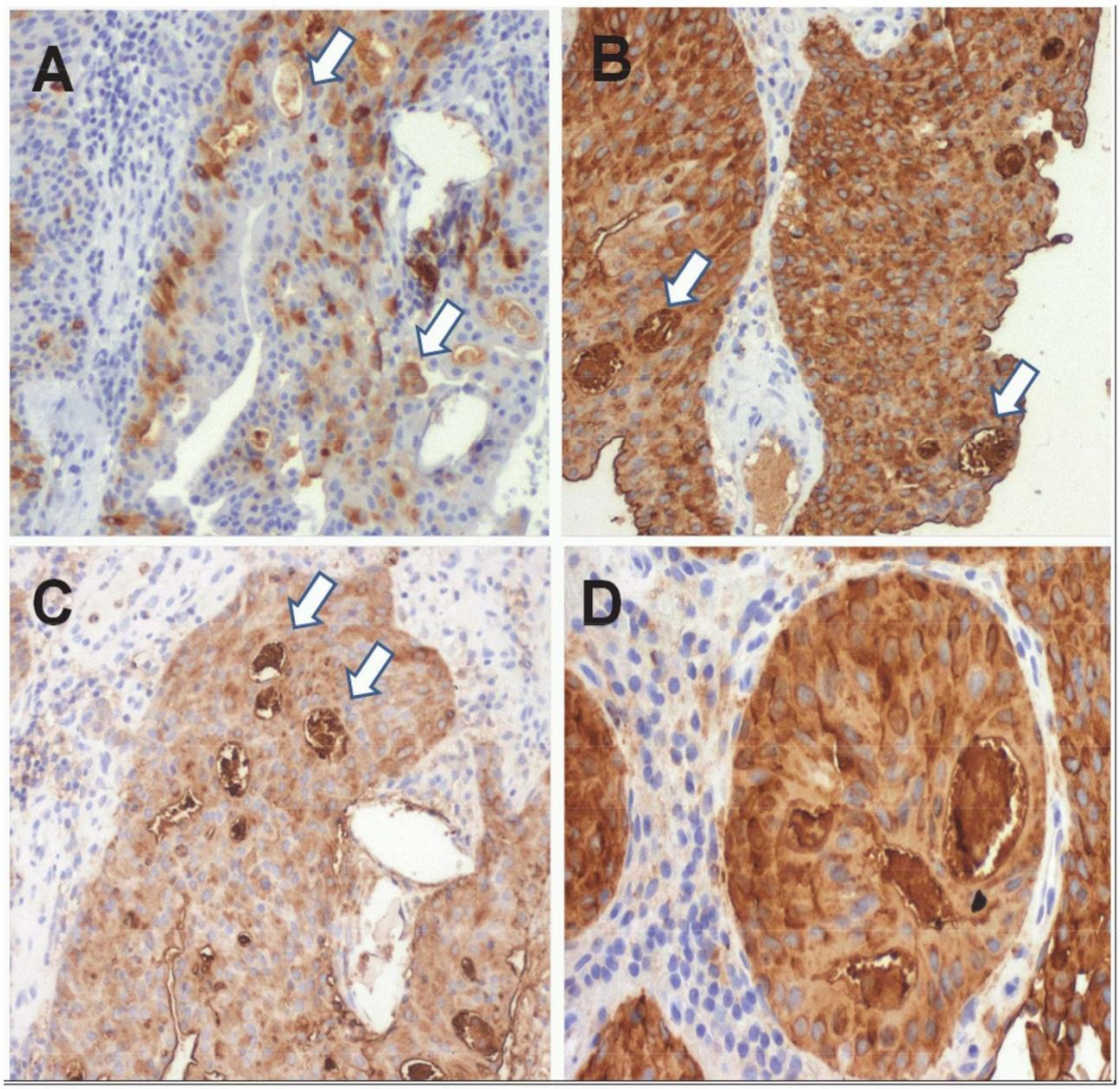

Fig. 2. Expression of cancer-associated glycans antigens A) sTn, B)sLea and C-D) sLe^x in *Schistosoma haematobium* eggs. The white arrows point to positive eggs. The treatment of the tissue sections with a α-neuraminidase led to the loss of immunoreactivity, confirming the validating the structural assignment.
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the null hypotheses are true, 5% of the comparisons are expected to present uncorrected *P* values lower than 0.05 by chance alone. However, our study present *P* values under 0.05 in 20% of the comparisons, thereby demonstrating the statistic value of the observations.

The expression of cancer-associated cell-surface sialylated glycans sTn, sLe^a and sLe^x was addressed, to our knowledge, for the first time in *S. haematobium*-associated bladder tumours. Glycosylation is the main and more complex posttranslational modification of membrane-bound and secreted proteins. Glycans plays a key role in protein folding and stability [43], mediate several physiological and pathological conditions, which include cell-cell adhesion, host-pathogen interactions, cell differentiation, migration and cell trafficking, signaling and immune recognition [44,45]. During malignant transformation, some cells change their glycosylation profile in response to microenvironment challenge, namely paracrine signaling, hypoxia among other events [29]. The sTn antigen, resulting from a premature stop in the *O*-glycosylation of proteins by sialylation, has been found associated with high-grade bladder non-muscle invasive papillary tumours and muscle invasive lesions [30,31]. It has been found to enhance bladder cancer cells capability to invade and migrate [31] and acts as a suppressor of effective dendritic cell immune responses against bladder cancer cells [46]. Despite the low number of cases, this study suggests that the sTn antigen is predominantly expressed by more aggressive forms of *S. haematiobum* associated tumours (UCC+SCC). These observations are in accordance with our previous results from non-schistosome associated bladder tumors, were sTn expression is predominant found in high grade papillary tumors and muscle invasive bladder cancer of non-schistosome

etiology [31]. The sialylated Lewis blood group determinants sLe^a and sLe^x may be found as terminal structures of both proteins and lipids and have been associated with metastatic potential and poor overall survival in several solid tumours [47–50]. The sLe^a antigen has also been found both in pre-malignant bladder lesions, non-invasive and invasive bladder urothelial carcinomas [32–34]; however no association with recurrence, invasion or metastasis has been reported. On the other hand, its structural isomer sLe^x antigen was observed in muscle invasive urothelial carcinomas associated with invasion, metastasis and recurrence [35,36]. This study now demonstrates that the majority of schistosome-associated bladder tumours expressed the sLe^x antigen, suggesting a high degree of malignant potential. However, no defined expression pattern could be drawn for sLe^a Similarly, we also found significant overexpression of the sLe^x antigen in non-schistosome associated tumours but also a more pronounced expression of sLe^a. However, none of these antigens were not observed in the healthy urothelium, reinforcing its malignant nature.

The analysis of benign/pre-malignant lesions, irrespectively of their origin, showed a predominance of p53, sLe^a and sLe^x positive cases. Noteworthy, sole lesions were predominantly non-proliferative when compared to lesions in the vicinity of tumours, suggesting that high proliferation may be mainly a characteristic of the tumour. Whether proliferative benign/pre-malignant lesions present a higher risk of evolving to bladder cancer warrants validation in future studies.

On the other hand, the sLe^a antigen was predominantly expressed among sole lesions, denoting this antigen may constitute a marker of early bladder carcinogenesis mediated by *S. haematobium*. Reinforcing this observations, Kajiwara et al. has described that sLe^a is inversely associated with the grade of atypia while its non-sialylated form DU-PAN-2 correlates with the grade of atypia in urothelial carcinomas; these authors also observed that the disappearance of sLe^a and the presence of DU-PAN-2 correlates with high malignant potential [36]. We further observed that the expression of cancer-associated antigens in the tumour was correlated with the expression denoting a field effect that affects the entire bladder. Again, this correlation was not observed for proliferation, reinforcing this event see ms to be mainly a characteristic of malignant lesions. Taken together, these observations highlight that pre-malignant lesions present molecular alterations associated with malignancy, and that p53 and sLe^x are surrogate markers of bladder cancerization associated with infection with schistosomes. More studies should be conducted to validate the potential of sLe^a has a surrogate marker of infection that may be helpful in the monitoring of asymptomatic colonization. A glycoproteomic/lipidomic characterization of bladder tumours is ongoing, which is expected to provide their necessary insights about the biologic role of the studied glycans in bladder cancer.

These observations are also likely to be of consequence in the clinic of great importance since benign and pre-malignant lesions such as those included in this study are challenging to diagnose by cystoscopy. We emphasize the potential of glycans in context, as they can be found at the cell-surface, thus easily accessible to antibodies and other carbohydrate ligands and consequently be explored in cancer detection imaging [51,52]. They are often secreted into the blood stream and urine and therefore readily accessible in non-invasive diagnosis [34,37, 38,53]. Non-invasive diagnostic procedures are critical as they facilitate large scale screening of the populations in endemic regions where imaging/radiological facilities are not likely to be available.

Glycans are also important mediators in the colonization of humans by parasites, as they provide means for efficient adhesion and immune escape [54,55]. As such, we have also addressed the expression of cancer-associated glycans in eggs of *S. haematobium* embedded in the bladders. We observed, for the first time, that the parasite eggs express sLe^a and sLe^x antigens, in mimicry of human leukocytes. These glycans are specific ligands for E-selectin, a cell adhesion molecule expressed only on endothelial cells and activated by cytokines, such as IL-1 and TNF-α, released by damaged cells during the course of inflammation [56,57]. Cytokines induce the overexpression of E-selectin by endothelial cells on nearby blood vessels that are responsible for recruiting leukocytes in a sLe^a/sLe^x-mediated manner [56,57]. These glycans bind weakly to E-selectin which allows leukocytes to "roll" along the internal surface of the blood vessel into the injury site by shear forces of blood flow [58]. Similar events may drive the recruitment of *S. haematobium* eggs to the bladder wall, a critical step in the developmental cycle of this pathogen. Similar strategies have been observed in nature, namely by the Gram-negative *Porphyromonas gingivalis* to adhere to human umbilical vein endothelial cells [59]. Several authors have also hypothesized that E-selectin-sialylated glycans interactions may contribute to the hematogenous dissemination of sLe^a/sLe^x expressing tumour cells and explain its association with metastasis [29,47,49,60]. Similarly, for bladder tumors, the identification of the parasite glycoproteins and/or glycolipids presenting these alterations may bring insights on this matter and ultimately contribute to design strategies to control infection. In addition, the identification of the glycoproteins and/or glycolipids presenting these alterations may yield insights into this infection-associated cancer and ultimately contribute to design strategies to control infection. Glycoproteomic studies will greatly benefit from the recent mapping of the parasite genome [61]. We further report that some eggs express the sTn antigen, an oncofetal antigen that, we and others have shown to play a key role in immune escape [46]. The sLe^x expression has also been found to reduce the susceptibility of tumour cells to hepatic sinusoidal lymphocyte-mediated killing, and thus, may facilitate the ability of the tumor cells to metastasize to the liver [62]. Similarly, the expression of these glycans by *S. haematobium* may provide the necessary means for immune escape either by modulation of the immune system or by molecular mimicry of the host, a common survival strategy among parasites [63,64].

To conclude p53 and sialylated Lewis blood group determinants may be surrogate markers of cancerization associated with chronic infection with *S. haematobium*. By drawing attention to common molecular pathways underlying these two events, this study provides one of the missing links associating parasite infection and cancer development. Further studies which include a larger sample of *Schistosoma haematobium* positive cases will be needed to determine a panel of biomarkers with the potential to identify bladder cancer precursor lesions Finally, this report provides insights on the glycosylation patterns of *S. haematobium* eggs and discusses a possible model for the recruitment of eggs to the bladder wall that suggests this schistosome has evolved glycosylation patterns that mimic those of its human host. These insights may help guiding the development of novel therapeutic strategy, namely glycoconjugate vaccines.

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Author Contributions

Conceived and designed the experiments: JS EF JAF LLS. Performed the experiments: JS EF AP AT JAF BP. Analyzed the data: JS EF JAF LL CL LLS. Contributed reagents/materials/analysis tools: JS JMCdC CL LLS. Wrote the paper: JAF LL PJB LLS. Revised the manuscript: JMCC CL LLS.

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Capítulo 6

Schistosome and liver fluke derived catechol estrogens and helminth associated cancers, in *Frontiers in Genetics*.

CAPÍTULO 6

Schistosome and liver fluke derived catechol estrogens and helminth associated cancers, *in Frontiers in Genetics*.

José M. Correiada Costa, Nuno Vale, Maria J. Gouveia, Mónica C.Botelho, Banchob Sripa, Lúcio L.Santos, Júlio H. Santos, Gabriel Rinaldi and Paul J. Brindley.

O artigo apresentado como Capítulo 6 tinha um propósito: estruturar uma visão integrada e postular sobre mecanismos moleculares implicados na carcinogénese associada a infeções por Helmintos. De facto, estas infeções continuam a ser um problema persistente de saúde pública nos países em desenvolvimento. Particularmente *Clonorchis sinensis*, *Opisthorchis viverrini* e *Schistosoma haematobium*, são de particular preocupação devido à sua classificação como carcinogéneos do grupo 1 pelo IARC/OMS, i.e., as infeções induzidas por esses vermes podem ser cancerígenos para os seus hospedeiros: *C. sinensis* e *O. viverrini* estão associados a colangiocarcinoma, o *S. haematobium* a carcinoma da bexiga. Utilizando o potencial analítico da Cromatografia Líquida associada a Espectrometria de Massa (LC-MS/MS), identificámos moléculas com capacidade de reagir com o ADN dos hospedeiros promovendo lesões; oxisteróis, derivados do colesterol, nos dois primeiros, e catecol estrogénios no *S. haematobium*. Porque essas moléculas derivadas de estradiol e de colesterol são metabolizados para formas ativas que podem modificar o ADN, induzindo a formação de fenótipos celulares tumorais nos hospedeiros, ou seja, nas células de urotélio da bexiga no caso da UGS e no epitélio dos ductos biliares, nos colangiócitos, nos casos de infeção por *O. viverrini* e *C. sinensis*, postulamos a visão aqui descrita.

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Schistosome and liver fluke derived catechol-estrogens and helminth associated cancers

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Infection with helminth parasites remains a persistent public health problem in developing countries. Three of these pathogens, the liver flukes *Clonorchis sinensis*, *Opisthorchis viverrini* and the blood fluke *Schistosoma haematobium*, are of particular concern due to their classification as Group 1 carcinogens: infection with these worms is carcinogenic. Using liquid chromatography-mass spectrometry (LC-MS/MS) approaches, we identified steroid hormone like (e.g., oxysterol-like, catechol estrogen quinone-like, etc.) metabolites and related DNA-adducts, apparently of parasite origin, in developmental stages including eggs of *S. haematobium*, in urine of people with urogenital schistosomiasis, and in the adult stage of *O. viverrini*. Since these kinds of sterol derivatives are metabolized to active quinones that can modify DNA, which in other contexts can lead to breast and other cancers, helminth parasite associated sterols might induce tumor-like phenotypes in the target cells susceptible to helminth parasite associated cancers, i.e., urothelial cells of the bladder in the case of urogenital schistosomiasis and the bile duct epithelia or cholangiocytes, in the case of *O. viverrini* and *C. sinensis*. Indeed we postulate that helminth induced cancers originate from parasite estrogen-host epithelial/urothelial cell chromosomal DNA adducts, and here we review recent findings that support this conjecture.

Keywords: urogenital schistosomiasis, opisthorchiasis, catechol-estrogens, oxysterols, DNA-adducts, neglected tropical disease-associated-cancer, squamous cell carcinoma of the bladder, cholangiocarcinoma

BIOLOGICAL CARCINOGENS – THREE HELMINTH PARASITES

The World Health Organization's International Agency for Research on Cancer (IARC) and the United States' National Institutes of Health (NIH) consider that ~20% of cancers are caused by infectious diseases. Some cancer-inducing infectious agents, such as Hepatitis B and C Viruses, are well known. However, less appreciated are the several major human helminth pathogens that cause cancer. IARC recognizes three helminth infections as definitive causes of cancer – the fish-borne liver flukes *Opisthorchis viverrini* and *Clonorchis sinensis* and the blood fluke *Schistosoma haematobium* (Bouvard et al., 2009; de Martel et al., 2012; International Agency for Research on Cancer (IARC), 2012; **Figure 1**). In addition to direct detriment on development and health of infected populations, infection with liver flukes and schistosomes – types of helminth parasites collectively termed trematode flatworms – lead to infection related cancers, specifically cholangiocarcinoma (CCA; bile duct cancer) and squamous cell carcinoma (SSC) of the urinary bladder, respectively.

UROGENITAL SCHISTOSOMIASIS AND BLADDER CANCER

Three major species of schistosomes are the agents of human schistosomiasis – *Schistosoma japonicum* and *Schistosoma mansoni* cause intestinal schistosomiasis in East Asia, Africa, South America, and the Caribbean while *S. haematobium*, occurring widely through Africa and the Middle East, causes urogenital schistosomiasis (**Figure 1**). In the range of 4.5–70 million disability adjusted life years (DALYs) are lost to schistosomiasis (King and Dangerfield-Cha, 2008). More people are infected with *S. haematobium* than with the other schistosomes. Of ~112 million cases of *S. haematobium* infection in sub-Saharan Africa, 70 million are associated with hematuria, 18 million with major bladder wall pathology, and 10 million with hydronephrosis leading to kidney damage (van der Werf et al., 2003; Hotez et al., 2009; King, 2010). In many patients, deposition of *S. haematobium* parasite ova eventually leads to SSC of the bladder (Hodder et al., 2000; Parkin, 2006). Moreover, as many as 75% of women infected with *S. haematobium* suffer from female genital schistosomiasis (FGS) of the lower genital tract (Hotez et al., 2009). FGS results from

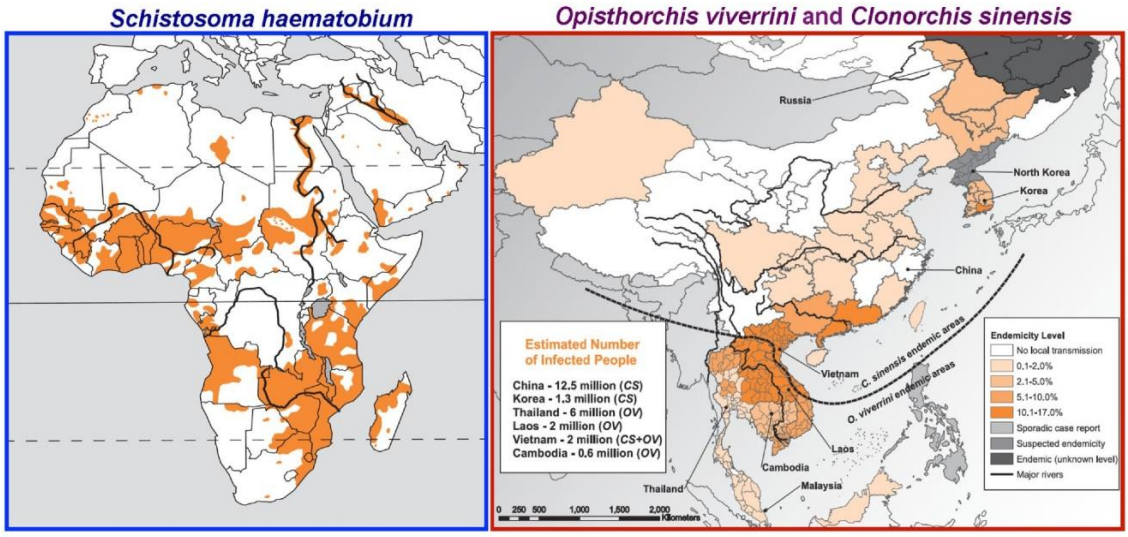

FIGURE 1 | Geographical distribution of the carcinogenic flukes. Left: Distribution of the blood fluke *Schistosoma haematobium* (sub-Saharan Africa, Nile valley in Egypt and Sudan, the Maghreb, and the Arabian peninsula). Right: Distribution of the liver flukes *Clonorchis sinensis* (China, Republic of Korea, Democratic People's Republic of Korea, the Russian Federation, and northern Viet Nam) and *Opisthorchis viverrini* (Thailand, Laos, Cambodia, Malaysia, and southern part of Viet Nam). "No local transmission" stands for "no reported local transmission." From International Agency for Research on Cancer (IARC), (2012); permission requested from WHO Press, International Agency for Research on Cancer.

deposition of schistosome eggs in the uterus, cervix, vagina and vulva, with ensuing inflammatory responses. It impairs fertility (Santos et al., 2014) and also increases susceptibility of the woman to HIV (Feldmeier et al., 1994; Kjetland et al., 2006; Ndhlovu et al., 2007; Jourdan et al., 2011).

Squamous cell carcinoma is a malignant, poorly differentiated neuroendocrine neoplasm. SCC is the common form of bladder cancer in rural Africa where *S. haematobium* is prevalent (Mostafa et al., 1999; Zhong et al., 2013). In contrast, the majority of bladder cancer in developing countries and regions not endemic for urogenital schistosomiasis is transitional cell carcinoma (TCC) that arises from the transitional epithelium lining of the bladder. The parasite eggs trapped in the bladder wall release antigens and other metabolites (presumably evolved to expedite egress to the urine, and hence to the external environment). Nonetheless, the phenomenon leads to hematuria and to chronic inflammation, in turn increasing risk of urothelial hyperplasia, dysplasia, and SCC of the bladder (Honeycutt et al., 2014). The epidemiologic association between SSC of the bladder with schistosomiasis haematobia is based both on case control studies and on the correlation of bladder cancer incidence with prevalence of infection with *S. haematobium* within different geographic locations. Schistosomiasis haematobia is a chronic infection, the adult, egg-producing schistosomes live for many years, re-infections frequently occur, and schistosomiasis associated bladder SCC appears relatively early, often by the mid-decades of life. By contrast, TCC usually presents in the later decades of life. The incidence of urogenital schistosomiasis associated SCC is estimated in 3–4 cases per 100,000 (Shiff et al., 2006).

FISH-BORNE FLUKES AND BILE DUCT CANCER

Liver infection caused by *O. viverrini*, *C. sinensis* and related flukes remains a major public health problem in East Asia and Eastern Europe where >40 million people are infected. *O. viverrini* is endemic in Thailand, Lao PDR, Vietnam and Cambodia (Sripa et al., 2011; Sithithaworn et al., 2012; **Figure 1**). Humans acquire the infection with *O. viverrini* by eating undercooked, fresh water cyprinoid fish infected with the metacercariae of the fluke (Sripa et al., 2011). There the parasites mature over 6 weeks into adult flukes, which graze on biliary epithelia. Eggs of *O. viverrini* are shed in bile and exit the infected person with the fecal stream. Freshwater snails ingest the eggs; the parasite (and related flukes, above) undergoes transformations within the snail host, culminating in the release of cercariae that seek out and penetrate the skin of a freshwater fish. Where sanitation is less than optimal, eggs may enter fresh water ecosystems where the eggs are ingested by freshwater snails. Human infection leads to hepatobiliary disease, cholangitis, obstructive jaundice, hepatomegaly, periductal fibrosis, cholecystitis, and cholelithiasis (Blechacz et al., 2011; Mairiang et al., 2012). More problematically, experimental and epidemiological evidence implicates liver fluke in the etiology of a major sub-type of liver cancer, CCA or bile duct cancer [Bouvard et al., 2009; de Martel et al., 2012; International Agency for Research on Cancer (IARC), 2012].

Cholangiocarcinoma, bile duct cancer, is an adenocarcinoma of the bile ducts, with a dismal prognosis. These are slow growing tumors, which spread along bile ducts with periductal and mass forming extensions. Prognosis is poor owing to the silent clinical character, difficulty in early diagnosis, and limited therapeutic approaches, especially in resource poor settings such

as northeastern Thailand where the recent estimate of median survival time after supportive treatment was 4 months (Thunyaharn et al., 2013). Surgical management is the only potentially curative treatment, but is restricted to early-stage disease. CCA has a worldwide distribution, beyond East Asia, where patients often develop CCA *de novo* without obvious risk factors. Primary sclerosing cholangitis and congenital bile duct anomalies are also precursors. In Thailand and elsewhere in East Asia, where infections with liver flukes are definitive risk factor, the factors share a common determinant of chronic inflammation and chronic injury of the biliary epithelium, including from persistent parasitism by these fish-borne trematodes (Sripa et al., 2009, 2012; Blechacz et al., 2011; Johnson et al., 2012; O'Hara et al., 2013; Razumilava and Gores, 2013).

FLUKES, CATECHOL-ESTROGENS, OXYSTEROLS, AND CARCINOGENESIS

In addition to the hormone-like effects of the parasite estradiol-related molecules on the endocrine and immune system of the host, initiation metabolites of estrogens can be also considered as carcinogenic chemicals (Cavalieri and Rogan, 2011, 2014). Hydroxylation of estrogens forms the 2- and 4-catechol estrogens involved in further oxidation to semiquinones and quinones, including formation of the catechol estrogen-3, 4-quinones, the major carcinogenic metabolites of estrogens. These electrophilic compounds react with macromolecules, including DNA, to form the depurinating adducts that eventually lead to mutations and cancer initiation (**Figure 2**; Cavalieri and Rogan, 2011). Several mechanisms explain the role of estrogens in disease. The better-known hypothesis is that the estrogen receptor mediates cell proliferation, increasing errors in DNA replication (Clemons and Goss, 2001; Yager and Davidson, 2006; Botelho et al., 2009b, 2013). Another interpretation postulates that estrogen metabolites react covalently with DNA bases by redox cycling or by forming an abasic site. Subsequent error-prone repair of the modified DNA generates oncogenic mutations that initiate cancer. The two mechanisms may act in concert. According to the second mechanism, P450 metabolism of estrone and estradiol generates the catechol estrogens, 2-hydroxyestrogen and 4-hydroxyestrogen. Further oxidation leads to 2, 3-catechol-

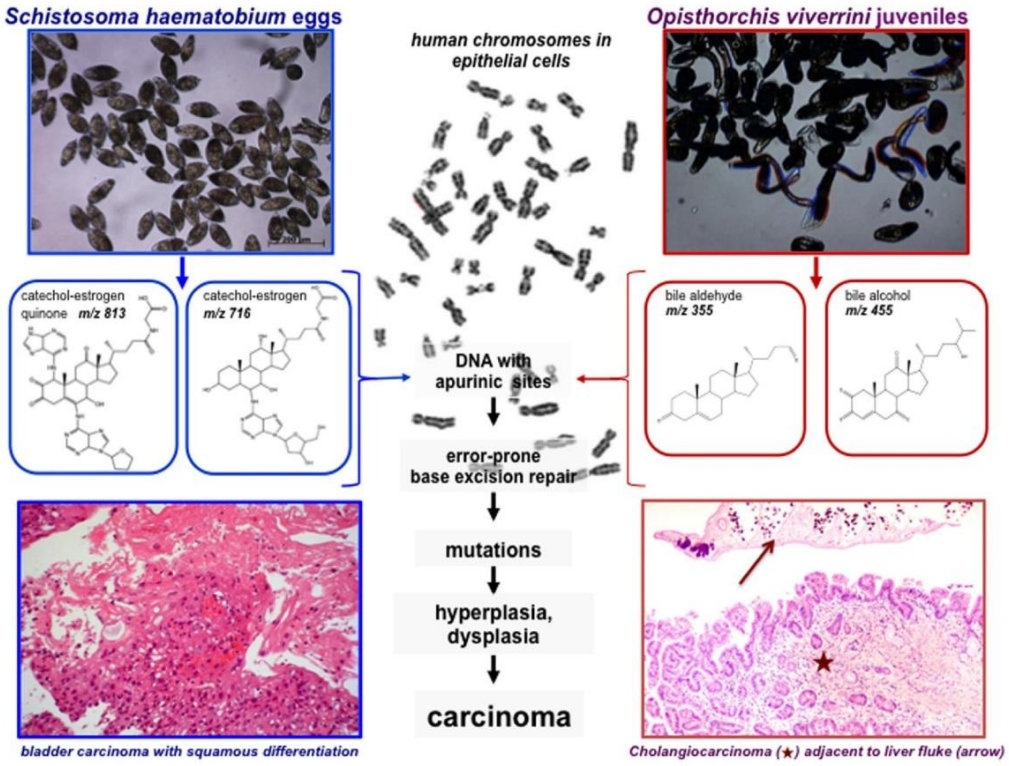

FIGURE 2 | Carcinogenesis mediated by steroid hormone like molecules derived from *S. haematobium* and *O. viverrini*. Eggs of *S. haematobium* derived catechol-estrogens and DNA-adducts (left; Botelho et al., 2013), and *O. viverrini* derived oxysterols (right; Vale et al., 2013), likely interact with the chromosomes of target cells inducing DNA apurinic sites that eventually escape the DNA repair mechanisms leading to mutations. These mutations ultimately would transform the target cell, leading to hyperplasia and ultimately to neoplasia, i.e., squamous cell carcinoma of the bladder [bottom left, brief description adapted from Santos et al. (2014)] and liver fluke induced cholangiocarcinoma (CCA; bottom right). Hematoxylin and eosin stained section of human intrahepatic bile duct/liver, cholangiocarcinoma at bottom of image (star) and an adult *O. viverrini* liver fluke (arrow) at top. Image contributed by co-author Banchob Sripa. Human metaphase chromosomes – image from Tang et al. (2013).

estrogen quinine and 3, 4-catechol-estrogen quinone, respectively, which can react directly with DNA via a Michael addition or indirectly via generation of reactive oxygen species. Methylation of catechol estrogens by catechol-*O*-methyltransferase, conjugation of the catechol estrogen quinones with glutathione, and enzymatic reduction to reform catechol estrogens are processes that prevent accumulation of the highly reactive metabolites. However, if the latter protective processes are insufficient, catechol estrogen quinones accumulate, which damage DNA either by oxidation or depurination, and release of catechol estrogen modified purines (Liehr et al., 1986; Cavalieri et al., 1997; Yager and Davidson, 2006).

While examining human cases of urogenital schistosomiasis from Angola, we observed elevation in levels of estradiol in sera but not luteinizing hormone (LH) or testosterone (Botelho et al., 2009a). Estradiol is a steroid hormone secreted principally by the ovarian follicles in vertebrates. It seemed implausible that the elevated levels could be attributed to hypothalamic–pituitary–gonadal axis regulation. Rather, we speculated that schistosomes produced the estradiol-related metabolites that contributed to the elevated estradiol levels. Using mass spectrometry approaches (Gouveia et al., 2013) we characterized >20 estradiol related metabolites, from sera of *S. haematobium*-infected persons from Angola and, remarkably, in the parasites including the eggs (Botelho et al., 2013). Catechol-estrogens are formed by hydroxylation on the steroid aromatic ring A. Hydroxylation of both C-2 and C-3 on a steroid ring was apparent and, further, oxidation to an estradiol-2,3-quinone. The schistosome estrogenic metabolites readily seen in urine and *in vitro* appear to arise by reactions of quinones of catechol estrogens with chromosomal DNA (Botelho et al., 2011b, 2013). In addition, we exposed non-cancerous CHO (Chinese hamster ovary) cells to secretions and lysates of *S. haematobium eggs* and adult parasites, which stimulated cellular proliferation, migration and invasion, inhibited apoptosis, up-regulated expression of *Bcl-2*, and facilitated loss of *p27* in CHO (Botelho et al., 2009a,b, 2010, 2011a,b, 2013) – processes that are hallmarks of tumorigenesis and cancer cell survival (Hanahan and Weinberg, 2000). If similar phenomena also occur in human urogenital schistosomiasis, we speculate that they contribute to the abnormal proliferation and accumulation of genetic changes that occur in schistosomiasis-associated carcinogenesis [**Figure 2**; Mostafa et al., 1999; International Agency for Research on Cancer (IARC), 2012].

Opisthorchiasis is associated with elevation of bile acids, including deoxycholic acid (Vale et al., 2013) which are potent tumor promoters in cholangiocarcinogenesis (Sirica, 2005). Bile acids are synthesized in the liver from cholesterol, and the majorities are conjugated with either glycine or taurine (Haswell-Elkins et al., 1994; Ohshima et al., 1994; Akaike et al., 2003; Pinlaor et al., 2003; Katsuma et al., 2005; Sirica, 2005; Yongvanit et al., 2012). Inflammation-related carcinogenesis has also been associated to oxidative and nitrative DNA damage as 8-oxo-7,8-hydro-2′-deoxiguanine (8-oxodG) and 8-nitroguanine (8-NG; Yongvanit et al., 2012). Increased levels of nitrate and nitrite, which reflect endogenous generation of NO, occur during *O. viverrini* infection in humans (Haswell-Elkins et al., 1994) and rodents (Ohshima et al., 1994). Oxysterols, which are oxidation products of cholesterol generated by enzymatic (P450) or non-enzymatic processes (Jaworski et al., 2001; Jusakul et al., 2011), can be mutagenic or genotoxic, and to possess pro-oxidative and pro-inflammation properties that promote carcinogenesis. Investigation of binding domains in human genes has demonstrated an association between different types of oxysterols and the development and progression of cancer of the colon, lung, breast and bile ducts (Jaworski et al., 2001). Bile acids constitute a large family of steroids carrying a carboxyl group in the side chain. Bile alcohols have similar products in bile acid biosynthesis or as end products. We found these compounds in extracts of *O. viverrini* (**Figure 2** compound 18) but conjugated at different positions, free bile acids re-conjugated in some species like aldehydes (**Figure 2** compound 12) or as sulfates (not shown). The effects of these individual species can be anticipated to be structure-dependent, and metabolic conversions will result in a complex mixture of biologically active and inactive forms (Vale et al., 2013).

INFECTION WITH BLOOD FLUKES AND LIVER FLUKES AS THE RISK FACTOR – BUT HOW MIGHT CANCER ARISE?

Current understanding of how infections with these flukes lead to cancers has been reviewed recently (Sithithaworn et al., 2012; Sripa et al., 2012; Honeycutt et al., 2014). In brief, in regions of high prevalence of opisthorchiasis, the risk factors for bile duct cancers are chronic inflammation and concomitant chronic injury of the biliary epithelium as the consequence of persistent parasitism by these fish-borne pathogens (Sripa et al., 2009, 2012; Blechacz et al., 2011; Johnson et al., 2012; O'Hara et al., 2013; Razumilava and Gores, 2013). The risk of SSC of the bladder during urogenital schistosomiasis appears to be promoted by concurrent risk factors associated with bladder cancer where infection with *S. haematobium* is less common or in non-endemic regions including exposure to toxins such as dyes from industrial and agricultural sources, and from tobacco smoke (see Honeycutt et al., 2014). Thus there are likely to be multiple factors including a diet rich in nitrosamines, spillover effects from local and systemic chronic inflammation (reactive oxygen species, reactive nitrogen species) directed against the worms, the secretion of mitogens and other mediators by the parasite (Satarug et al., 1998; Sripa et al., 2012), and interactions or changes in the biliary, GI tract and urinary tract microbiota, including co-infection by other potentially oncogenic biological species (Plieskatt et al., 2013).

To this list, we now include another potential mechanism: lesions in chromosomes and production of depurinating estrogen-DNA adducts leading to parasite metabolite-promoted host cell DNA damage, due to parasite-derived, reactive oxysterol and/or catechol estrogen derivatives. These processes contribute to urogenital schistosomiasis associated SCC during chronic urogenital schistosomiasis, and to CCA during chronic opisthorchiasis (**Figure 2**). Overall, the structures that we have identified in *S. haematobium* and *O. viverrini* (Botelho et al., 2013; Vale et al., 2013) suggest that carcinogenesis-related steroids may be released in carcinogenic quantities by these flukes. Notably, a relation between putative oxysterol or bile acid metabolites from *O. viverrini* and bile duct cancer has long been hypothesized (Changbumrung et al., 1990).

CONCLUDING COMMENTS

Infection with helminth parasites remains a persistent public health problem in developing countries. Three of these pathogens, *C. sinensis*, *O. viverrini*, and *S. haematobium*, are of particular concern due to their classification by the IARC as Group 1 carcinogens. Infection with these worms is definitively associated with cancer. We have reported novel sterol-like metabolites and DNA-adducts in *S. haematobium*, in urine of persons with urogenital schistosomiasis, and in *O. viverrini*. Because these molecules are metabolized to active quinones that can modify DNA, helminth parasite associated catechol estrogens might induce tumor-like phenotypes in the epithelia of the bile ducts and bladder. Whereas the roles of these new metabolites in bile duct cancer and SSC of the bladder remain to be examined in depth, this clearly is worthy of deeper investigation. Future studies might profitably aim for isolation or chemical synthesis of these putative carcinogens and downstream investigation of interactions of the fluke estrogens and oxysterols with informative cells such as bladder urothelial cells (Botelho et al., 2013) and cholangiocytes (Grubman et al., 1994), and with oxysterol binding proteins and so forth. The interrelations of these carcinogens and the microbiota of the infected bladder and biliary system can also be predicted to be informative (Plieskatt et al., 2013). Moreover, given that other metabolites of *O. viverrini* are predicted to play a role in carcinogenesis of *O. viverrini* induced bile duct cancer, including liver fluke granulin (Smout et al., 2009), it will be informative also to compare and contrast action of liver fluke granulin and other fluke metabolites in these analyses, investigations that are now facilitated by the availability of genome sequences of these carcinogenic flukes (Wang et al., 2011; Young et al., 2012, 2014; Brindley and Hotez, 2013; Huang et al., 2013), genome sequences of CCAs (Chan-On et al., 2013), new rodent models (Fu et al., 2012), and functional genomic approaches developed for these parasites (Rinaldi et al., 2011, 2012). In addition to their carcinogenic effects, these flukes-associated sterol derivatives and DNA-adducts could be exploited as diagnostic and prognostic biomarkers, indeed 8-oxo dG in urine associates with opisthorchiasis-induced CCA (Thanan et al., 2008), and as targets for novel intervention strategies against these neglected tropical disease-associated cancers.

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Capítulo 7

Discussão geral, conclusões, e perspetivas.

CAPÍTULO 7:

Discussão geral, conclusões, e perspetivas.

Discussão Geral

Três espécies de *Schistosoma* são dominantemente os agentes etiológicos da schistosomose humana: *S. japonicum* e *S. mansoni*, que causam a schistosomose intestinal no sudeste da Ásia, África, América do Sul e Caraíbas, e o *S. haematobium*, que ocorre em África e no Médio Oriente e provoca a schistosomose urogenital (UGS). Excetuando a infeção por *S. japonicum*, todas as outras formas clínicas têm impacto na Saúde Pública de Angola. Historicamente consideradas patologias restritas às regiões tropicais e subtropicais, favorecidas pelo profusão de habitats adequados à transmissão dos agentes etiológicos, têm vindo recentemente a expandir-se para a Europa Ocidental (Gautret P et al., 2015). Diversos casos têm sido assinalados em França, Alemanha e Itália. Aparentemente todos ligados ao mesmo foco endémico, um rio na ilha mediterrânica da Córsega (Holtfreter MC et al., 2014, Berry a et al., 2014; Beltrame A et al., 2015). O *S. haematobium* é o agente etiológico sistematicamente associado aos novos casos clínicos de schistosomose na Europa. Aparentemente, confirma-se o seu regresso ao continente europeu após a descrição do último caso autóctone em Portugal em 1965 (Fraga de Azevedo et al., 1969). Na presente tese circunscrevemos o nosso interesse maior à schistosomose urogenital e, particularmente, à sua associação com o carcinoma da bexiga. Já o referimos, apenas *S. haematobium* foi classificado pelo IARC/OMS como um carcinogéneo do grupo 1. Nos capítulos precedentes apresentámos os resultados deste exercício sob a forma de artigos publicados em revistas nacionais e internacionais com arbitragem. Com detalhe apropriado, apontámos questões científicas subjacentes, descrevemos os materiais e métodos utilizados, os resultados obtidos e procedemos a um discurso comparativo dos mesmos com referências na literatura. Agora propomos uma discussão mais abrangente, integrando elementos, antes parcelares, e enfatizando originalidades produzidas, visando melhorias na compreensão da schistosomose urogenital em Angola, na sua monitorização clínica, como contributo para o seu controlo no país. Infeção por S. *haematobium* e carcinoma da bexiga em Angola; quais as formas histológicas mais prevalentes e como se distribuem? Procedemos, por isso, a uma análise retrospectiva de 145 doentes (Capítulo 2). Foi um exercício prioritário, sensato, no contexto de uma região com limites conhecidos no registo do cancro; dele derivou a constatação de uma predominância do carcinoma espinocelular da bexiga associado à infeção por *S. haematobium*, já por demais reconhecida e amplamente descrita. No entanto, e ao contrário do referido por muitos autores egípcios, as mulheres

são preponderantes no grupo com carcinoma espinocelular. Salvo melhor explicação que o futuro nos possa trazer, julgamos que são os diferentes processos na organização social do trabalho entre o Egipto e Angola que estão na base desta substantiva diferença. A exposição da mulher Angolana à infeção será maior, porventura. Carlos Lopes foi o primeiro a enfatizar esta realidade em Angola, ainda no início dos anos oitenta (C. Lopes, 1985) O controlo da schistosomose urogenital em Angola deve assentar na quimioterapia com praziquantel, consensualmente segura e eficaz, no fornecimento de água potável às populações, no saneamento de cidades e aldeias, na melhoria dos níveis de educação e de higiene pública e no controlo das populações de moluscos-vetores. É um esforço considerável que desafia, recursos financeiros de vulto e competências médicas e de saúde adequadas. Perguntámo-nos muitas vezes, num cenário de poucos recursos, com inúmeras limitações, como melhorar a eficiência dos médicos e outros profissionais de saúde nos serviços de saúde locais na prestação de cuidados às populações nas áreas endémicas de UGS? No capítulo 3 damos conta de um exercício interessante de validação de instrumentos de diagnóstico imagiológico e endoscópico (ecografia e cistoscopia) na monitorização clínica da UGS. Podem as lesões causadas pela infeção de *S. haematobium* ser detetadas no exame ecográfico? O nosso estudo avaliou o desempenho da ultrassonografia nesse exercício, num grupo de pacientes na aldeia de Pita, e por comparação com os resultados obtidos na pesquisa da hematúria, e dos exames parasitológicos diretos, nos mesmos pacientes. Os resultados observados e descritos indicam inequivocamente que a ecografia (US) é adequada na monitorização clínica da UGS, constituindo-se como um precioso auxiliar no estudo da patologia do trato urinário, nomeadamente na sua evolução pós terapêutica. Outros autores enfatizaram a importância do exame US na avaliação de distúrbios estruturais associados à UGS; são claras as evidências de anormalidades pieloureterais causando estenoses e demonstra boa resolução das lesões da bexiga após o tratamento, inclusive em pacientes pediátricos. No entanto, a sua utilização em larga escala nos serviços de saúde locais deverá ser precedida por uma intensa fase de treino dos profissionais de saúde. Além disso, os dispositivos portáteis de US poderão ser úteis na avaliação de outras patologias de incidência, ginecológica e urológica.

A associação entre a infeção por *S. haematobium* e o cancro da bexiga foi primariamente referida no Egipto em 1911 (Ferguson, 1911). A Ferguson se adicionou evidências científicas posteriores baseadas em argumentos epidemiológicos, histológicos e mais recentemente moleculares: os primeiros enfatizaram que a incidência de cancro da bexiga no Médio Oriente e em África é maior em áreas com elevada prevalência da infeção; mais ainda, a prevalência global da infeção por *S. haematobium* no Egito é de 37 – 48%, e o cancro da bexiga apresentava valores de 31% da incidência total dos cancros.

Originalmente, numa meta análise de 2010, Salem e colaboradores, no Egipto, verificaram uma correlação muito significativa entre a diminuição da quantidade de ovos eliminados, por eficácia da quimioprofilaxia preventiva em massa, e a diminuição da incidência de cancro da bexiga; mais ainda, o perfil histológico dos carcinomas da bexiga no Egipto mudava, aproximando-se do perfil histológico de carcinoma da bexiga característico dos países desenvolvidos. No segundo argumento, vários autores relevam a maior incidência de carcinoma espinocelular da bexiga mas áreas endémicas de UGS. Por fim, os argumentos moleculares, onde situamos parte substancial da nossa atividade; a carcinogénese envolve distintos efeitos iniciais e sequenciais nas células alvo: iniciação, promoção e progressão. O primeiro envolve dano genético, que, a não ser reparado, leva a mudanças irreversíveis na cadeia complementar de ADN produzida durante a fase S do ciclo celular, originando mutação na célula somática. No capítulo I indiciámos alguns modelos postulados sobre carcinogénese vesical associada à infeção por *S. haematobium*; no Capítulo 6 conferimos, por hipótese, a responsabilidade, pelo menos parcial, a metabólitos de estrogénio produzidos pelo parasita na iniciação da carcinogénese; a nossa convicção sobre esta hipótese radica nos resultados obtidos no nosso trabalho e descritos nos Capítulos 4 e 5. Estes sucedem à resposta obtida a uma questão fundamental: podem os antigénios de *S. haematobium* induzir a tumorigénese? A resposta foi afirmativa: como referido previamente, células CHO tratadas em cultura celular com antigénio total de verme adulto de *S. haematobium* mostraram maior proliferação comparativamente com os controlos; mais, estas células, CHO tratadas, após inoculação via subcutânea, num modelo murino NUDE/NUDE, formaram sarcomas. As células dos sarcomas murinos expressaram filamentos de vimenctina e foram negativas para citoqueratina; os resultados demonstraram pela primeira vez que antígenos de *S. haematobium* induzem o desenvolvimento de tumores num modelo experimental animal (Botelho et al., 2009). Noutro estudo, já referido, verificou-se, após tratamento de células com os referidos antigénios, o aumento da sua proliferação, e da fase S do ciclo celular, e a diminuição da apoptose, bem como a diminuição da regulação da proteína supressora de tumores, p27, e maior regulação da molécula anti apoptótica, BCL-2, nas células tratadas com antigénios parasitários, por comparação com controlos. Aparentemente, o parasita desempenha um papel relevante na carcinogénese vesical. Posteriormente foi postulado que os mecanismos moleculares subjacentes à iniciação da carcinogénese estavam ligados à atividade de metabólitos de estrogénio (Correia da Costa et al., 2014). Uma questão se tornou pertinente, em consequência: quais e como são excretados na urina de pacientes com UGS os metabólitos de estrogénio? Poderemos antecipar alguma relevância destes metabólitos como biomarcadores da evolução para cancro da bexiga nos referidos pacientes? A este propósito os resultados

descritos no Capítulo 4 são claros: i) existem metabólitos de estrogénios específicos do *S. haematobium*, diferentes dos estrogénios endógenos; ii) os picos cromatográficos correspondentes a estes metabólitos são em maior número e mais elevados nos pacientes com UGS não associada a cancro; iii) o mesmo foi verificado relativamente à presença da molécula 8-oxo-dG. No grupo de pacientes com UGS não associado a cancro são espectáveis quer atividade inflamatória mais ativa, quer uma atividade de oxidação do ADN aumentada – parece ser este o sentido a extrair dos resultados obtidos. Por outro lado, no Capítulo 5, e utilizando o mesmo grupo de pacientes, agora após estudo parcimonioso das suas biópsias, um facto emergiu: a p53 detetada, por imunohistoquímica utilizando o anticorpo DO7, é profusamente expressa na mucosa urotelial vesical de doentes com UGS sem cancro vesical, bem como na mucosa urotelial vesical de doentes UGS com cancro da bexiga. Qual é o significado deste achado? Em primeiro lugar é fundamental verificar/comparar os nossos achados com o padrão mais frequente da expressão da p53 no urotélio normal da bexiga; da literatura decorre que, na mucosa urotelial normal, a p53 é raramente expressa; quando ocorre, a sua expressão é nuclear, pouco intensa e dispersa em células, predominantemente localizadas na região basal. Vários estudos realizados em mucosas uroteliais normais demonstraram que a p53 é expressa entre 1 e 37% dos casos em apenas 5% das células uroteliais e de forma dispersa. Em síntese; a expressão desta proteína em situações fisiológicas é rara e fugaz. (http://www.nature.com/modpathol/journal/v16/n3/pdf/3880741a.pdf;
http://www.ncbi.nlm.nih.gov/pubmed/11474293.
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2033449/pdf/brjcancer00047-0027.pdf).
Como já referido, a p53 controla os genes implicados na reparação do ADN e na ativação da apoptose. Alterações no ADN induzem a transcrição do gene *TP53* (gene supressor) localizado no braço curto do cromossoma 17 (17p13.1) e consequentemente a síntese da proteína p53. A p53 promove a interrupção do ciclo celular permitindo a reparação do ADN. Se as lesões não forem passiveis de reparação a apoptose é ativada; os dois mecanismos em consonância, reparação e apoptose, previnem a propagação de erros no ADN e controlam a sua qualidade. (Ver Anexo 1).
A p53 é degradada pela via metabólica da ubiquitina. A função da ubiquitina é sinalizar as proteínas que devem ser degradadas através da via proteolítica da ubiquitina-proteossoma (via específica responsável pela degradação e regulação de grande parte das proteínas de vida curta). Neste contexto, a expressão e atividade da p53 apresenta uma semivida curta. Os níveis de p53 são regulados pela MDM2, num processo alostérico. O MDM2 atua como uma ligase ubiquitina - E3, cooperando com p300/CBP na ubiquitinação e subsequente degradação proteossómica de p53 no citoplasma e núcleo, mas também controla a transcrição do gene *TP53*. A perda da função da p53 permite que

ocorra proliferação celular desordenada, longevidade celular e resistência a drogas citotóxicas. As alterações funcionais deste gene supressor (*TP53*) decorrem, grosso modo, de mutações que, para além de reduzirem a concentração da MDM2, favorecem a transcrição e a fosforilação da proteína, p53, e estão na base da perda da capacidade da p53 de promover a transcrição dos genes atrás referenciados. A p53 normal é, já o referimos, degradada rapidamente após a sua síntese; o mesmo não é verificado na forma dita mutada, alterada, da p53 que apresenta uma semivida aumentada, favorecendo a sua acumulação intracelular. A expressão da p53 nos carcinomas uroteliais é intensa e envolve clones celulares tanto de células malignas como de células uroteliais da mucosa adjacente, aparentemente normais (Palmeira C et al., 2011; Santos L et al., 2003; Santos LL et al., 2003). A expressão da p53 nas células do urotélio, detetada por anticorpos monoclonais – IHQ - e a ocorrência de mutações do gene *TP53*, em estudos comparativos, sugerem que a expressão profusa da p53 está associada a mutações do gene *TP53* (Baas IO et al., 1994; Gao JP et al., 2000; Vet JA et al., 1995; Khaled HM et al., 2003; Costa A et al., 1995; Soong R et al., 1996; Stoehr R1 et al., 2002). A expressão da p53 foi estudada em neoplasias malignas associadas ou não à infeção por *S. haematobium* (uroteliais e espinocelulares) verificando-se que em relação à taxa de expressão não existiam diferenças significativas em ambas as situações. A expressão da p53 era significativamente maior nos tumores pouco diferenciados porém, a expressão da p53 predominou nos tumores localmente avançados no grupo de UGS. (http://www.webio.hu/por/2006/12/3/0173/0173a.pdf). Badawi, e colaboradores, 1996, verificaram que, em pacientes com UGS, ocorria um número elevado de lesões do ADN associadas à ação de agentes alquilantes. As mutações ocorriam em simultâneo com perturbações dos mecanismos de reparação do ADN; mais, descreveu que essas mutações eram devidas a transições G-A no gene *H-ras* e nas sequências CpG do gene *TP53*. Finalmente, Abdulamir e colaboradores, 2009, verificaram que a expressão da proteína p53 era mais frequente em tumores malignos associados à UGS do que noutros tumores da bexiga; ainda, de acordo com Kamel D e colaboradores, 1994, em nenhum caso de hiperplasia e metaplasia escamosa em bexigas, não associado a UGS, revelou expressão aumentada da p53; situação inversa era referida nos casos de UGS. Os nossos resultados expressos no Capítulo 5 sustentam estas evidências. Na série de doentes com UGS por nós estudada, a expressão da p53, detetada por imunohistoquímica, era intensa, envolvendo um número elevado de células contiguas, sugerindo acumulação da p53 a nível nuclear. Esta situação biológica ocorre quando o gene *TP53* está mutado e não existe degradação da proteína. Nestas circunstancias a função da p53 está perturbada o que permite a acumulação de alterações no ADN e a sua transmissão às células filhas. Na ausência de reparação e apoptose, novas

mutações em genes críticos podem ocorrer. A regulação da proliferação celular é também perturbada. Cria-se assim o ambiente necessário e suficiente para que ocorra a transformação maligna. Na nossa amostra, repetimos, de pacientes com UGS, a expressão aumentada da p53 foi verificada em casos com cistite sem tumor associado, em casos com tumores malignos, espinocelulares, uroteliais ou mistos, e em casos com mucosas aparentemente normais adjacentes ao tumor. Em síntese; a concatenação de todos estes eventos moleculares, no grupo de pacientes estudados, sustenta, do nosso ponto de vista a atribuição de um papel relevante ao *S. haematobium* na iniciação da carcinogénese vesical, mediado por metabólitos de estrogénio. Finalmente, os resultados obtidos são francamente encorajantes no que se refere à possibilidade de definir num futuro próximo uma geração de novos marcadores urinários quer da schistosomose urogenital, e sobretudo da sua evolução para carcinoma da bexiga.

Conclusão

No presente exercício doutoral afigura-se-nos como possível concluir:

1) A Schistosomose Urogenital é um problema de Saúde Pública relevante em Angola; o carcinoma espinocelular é a forma histológica dominante dos tumores da bexiga, e pode aparecer na segunda e terceiras décadas de vida.
2) As mulheres em Angola são um alvo importante da UGS. A excessiva exposição à infeção por *S. haematobium* torna-as reféns de formas graves da UGS, particularmente do carcinoma espinocelular ou epidermoide e da schistosomose genital (Ver Anexo 2, artigo in *PLoS ONE* sobre infertilidade feminina em Angola).
3) Na prestação de cuidados primários de saúde em Angola o controlo da UGS é prioritário em muitas regiões, e tem implicações claras no registo e na monitorização do cancro; é urgente direcionar a formação das competências dos profissionais, incluindo a utilização da ecografia, nesta dupla vertente, favorecendo a colaboração e a interação mútuas.
4) Metabólitos de estrogénio previamente descritos em ovos e vermes adultos de *S. haematobium* e soros de pacientes com UGS foram agora identificados em amostras de urina de doentes com UGS; mais, alguns destes compostos só ocorrem neste grupo de doentes; tal facto indicia uma via metabólica de estrogénios específica do parasita; a sua associação com 8-oxodG na excreção urinária indicia uma putativa ação oxidante desses metabólitos no ADN do hospedeiro.

5) O gene *TP53* é um alvo frequente e precoce em casos de infeção por *S. haematobium*. As alterações neste gene podem ter um papel relevante no processo de cancerização vesical associadas à infeção.

Perspetivas.

No presente exercício, reportamos resultados de análises por LC-MS/MS realizadas em amostras de urina de 40 angolanos diagnosticado com UGS, metade dos quais apresentava UGS-associado, carcinoma espinocelular ou carcinoma urotelial. Os cromatogramas obtidos revelaram a presença de inúmeros metabólitos de estrogénio, incluindo sete especificamente identificada nos casos de UGS, por comparação com um banco de dados de metabólitos na urina de seres humanos saudáveis. Estes metabólitos associados a UGS incluem moléculas de catecol estrogénio (CEQ) e CEQ-ADN-aductos, dois dos quais tinham sido identificadas anteriormente em vermes adultos e ovos de *S. haematobium*. Além disso, a presença da molécula 8-oxodG, foi identificada na urina de todos os 40 casos de UGS, com diferenças substanciais entre o grupo de doentes UGS com cancro e sem cancro. O estudo posterior do comportamento e metabolização destes compostos pode perspectivar melhorias na compreensão dos mecanismos moleculares implicados na carcinogénese do carcinoma da bexiga induzida pela UGS, e como biomarcadores para diagnóstico e/ou prognóstico de cancro. Agora num número maior de pacientes, focalizados em premissas de sustentação do nosso postulado (metabólitos de estrogénio, 8-oxodG e outras moléculas indicadoras de lesão do ADN, mutações do gene *TP53* etc., e da proteómica urinária) e inseridos num programa que desejamos mais vasto que objetive o controlo da Schistosomose Urogenital em Angola.

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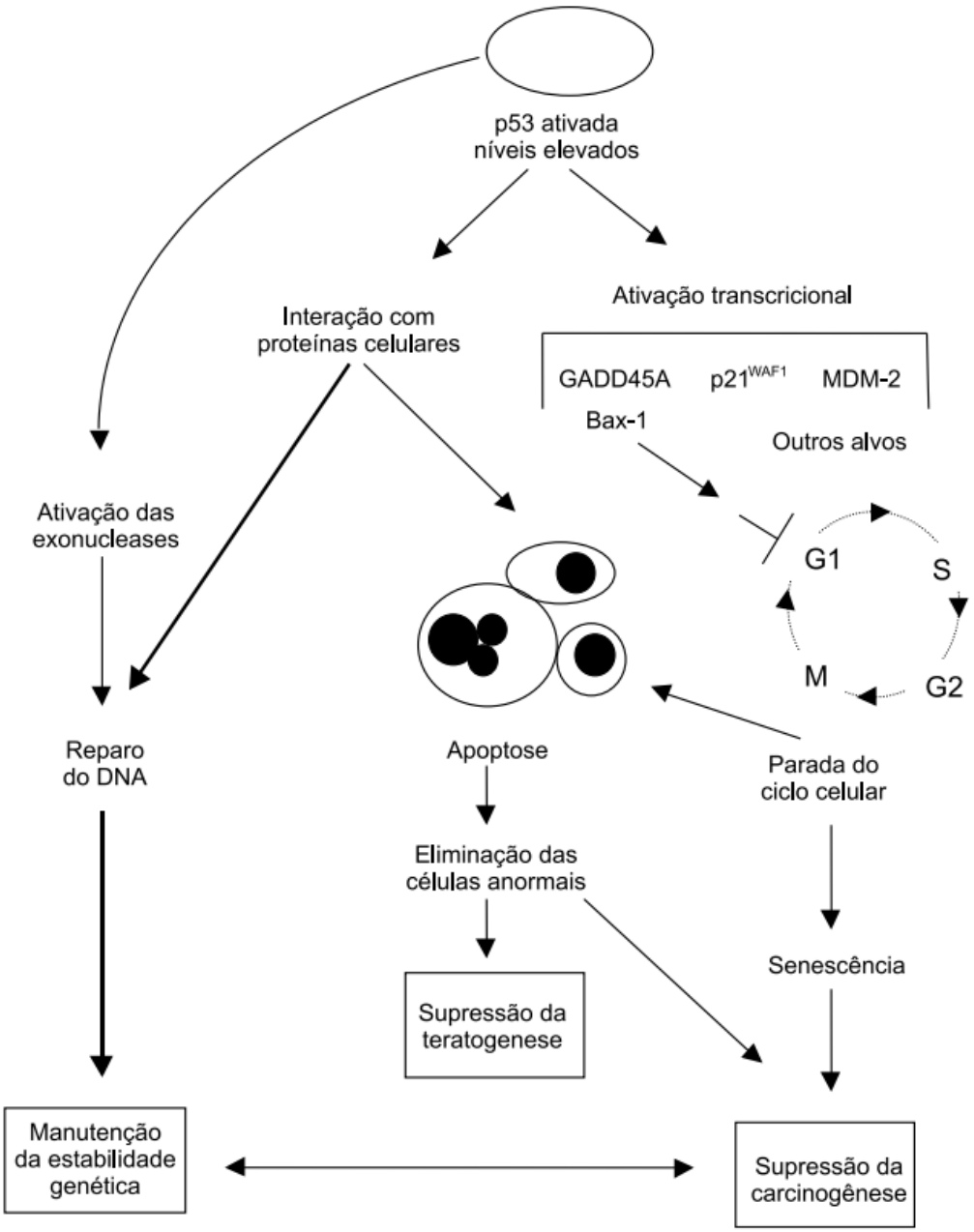
Anexos

ANEXO 1



Heterogeneidade das vias de sinalização utilizadas pela proteína p53 em seu papel de "guardiã do genoma" in Klumb C.E. et al, Rev.bras.hematol.hemoter.,2002,24(2):111-125

ANEXO 2

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PLOS ONE

Urinary Estrogen Metabolites and Self-Reported Infertility in Women Infected with *Schistosoma haematobium*

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Abstract

Background: Schistosomiasis is a neglected tropical disease, endemic in 76 countries, that afflicts more than 240 million people. The impact of schistosomiasis on infertility may be underestimated according to recent literature. Extracts of *Schistosoma haematobium* include estrogen-like metabolites termed catechol-estrogens that down regulate estrogen receptors alpha and beta in estrogen responsive cells. In addition, schistosome derived catechol-estrogens induce genotoxicity that result in estrogen-DNA adducts. These catechol estrogens and the catechol-estrogen-DNA adducts can be isolated from sera of people infected with *S. haematobium*. The aim of this study was to study infertility in females infected with *S. haematobium* and its association with the presence of schistosome-derived catechol-estrogens.

Methodology/Principal Findings: A cross-sectional study was undertaken of female residents of a region in Bengo province, Angola, endemic for schistosomiasis haematobia. Ninety-three women and girls, aged from two (parents interviewed) to 94 years were interviewed on present and previous urinary, urogenital and gynecological symptoms and complaints. Urine was collected from the participants for egg-based parasitological assessment of schistosome infection, and for liquid chromatography diode array detection electron spray ionization mass spectrometry (LC/UV-DAD/ESI-MSn) to investigate estrogen metabolites in the urine. Novel estrogen-like metabolites, potentially of schistosome origin, were detected in the urine of participants who were positive for eggs of *S. haematobium*, but not detected in urines negative for *S. haematobium* eggs. The catechol-estrogens/ DNA adducts were significantly associated with schistosomiasis (OR 3.35; 95% CI 2.32–4.84; $P \leq 0.001$). In addition, presence of these metabolites was positively associated with infertility (OR 4.33; 95% CI 1.13–16.70; $P \leq 0.05$).

Conclusions/Significance: Estrogen metabolites occur widely in diverse metabolic pathways. In view of the statistically significant association between catechol-estrogens/ DNA adducts and self-reported infertility, we propose that an estrogen-DNA adduct mediated pathway in *S. haematobium*-induced ovarian hormonal deregulation could be involved. In addition, the catechol-estrogens/ DNA adducts described here represent potential biomarkers for schistosomiasis haematobia.

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Introduction

At least 243 million people are infected with schistosomes, and more than half of these cases are caused by *Schistosoma haematobium*, the causative agent of urogenital schistosomiasis [1,2,3]. Indeed the number of cases of *S. haematobium* may be much greater than previously believed - perhaps as many as triple that of earlier

estimates of prevalence [4]. If confirmed, urogenital schistosomiasis may represent the most common neglected tropical disease in sub-Saharan Africa [5]. The adult stages of the blood flukes are long-lived within the venous plexi draining the pelvic organs including the urinary bladder, uterus, vagina, seminal vesicles and prostate [6]. The terminal spine eggs released continuously from the female schistosomes migrate from the circulation by breaking through the endothelial cells lining the venules. Thereafter, they traverse the wall of the urinary bladder to the lumen from where they exit to the exterior environment with the urine, to complete the transmission of this neglected tropical disease pathogen.

Problematically, many schistosome eggs fail to exit the body, and lodge with interstitial tissues of these organs. Entrapped eggs of *S. haematobium* induce granulomata and may be identified as various clinical presentations of 'sandy patches' and other signs and symptoms [3]. Hematuria is caused by the inflammation induced by entrapped eggs in the bladder and ureters. In addition, organ damage can follow obstruction of the ureters, as can secondary urinary tract and renal infections, hydronephrosis and renal failure [3]. Moreover, eggs of *S. haematobium* are biological carcinogens, grouped with a dozen or so other Group 1 microbes by the World Health Organization's (WHO) International Agency for Research on Cancer [7]. Chronic exposure to the eggs of *S. haematobium* frequently leads to squamous cell carcinoma of the bladder (SCC), the incidence of which is highest in regions endemic for *S. haematobium* [8,9]. Indeed, SCC is one of the most serious complications of chronic schistosomiasis haematobia [4,8,9,10,11]. In addition, the schistosomiasis haematobia likely influences endocrine homeostasis based in part on its ability of the blood flukes to synthesize and release estradiol [12,13].

We reported that extracts of *S. haematobium* worms include estrogen-related metabolites that down-regulate estrogen receptors (ER) alpha and beta in estrogen responsive cells *in vitro* [13]. Further, we identified estrogen metabolites in *S. haematobium* eggs by mass spectrographic analysis and also detected these schistosome-derived estrogens in sera of *S. haematobium*-infected persons. These estrogen-like metabolites belong to the catechol-estrogen family of molecules [14]. Estrogens arise by aromatization of androstenedione and testosterone, catalyzed by cytochrome P450 (CYP) 19, aromatase. They are metabolized by two major pathways – (1) formation of catechol estrogens and (2) 16α-hydroxylation [15] (Fig. 1). The natural estrogens, estrone (E1), and estradiol (E2), are metabolized at the 2- or 4-position with the formation of catechol estrogens that, in turn, are metabolically oxidized into catechol estrogen quinones (CEQ). CEQ have been implicated in the etiology of cancers [16]; the interaction of CEQ, in particular CE-3,4-Q, with DNA produces depurinating adducts, which may generate DNA apurinic sites that, in turn, initiate mutations [17]. In the catechol pathway, the metabolism involves further oxidation to semiquinones and quinones, including formation of the catechol estrogen-3,4-quinone, the major mutagenic derivative of estrogen [18]. Estrogen-derived metabolites associated with carcinogenesis in diverse tissues including the breast and thyroid [18,19,20] might impact target cells either as hormones (hence affecting gene expression regulation) and/or as carcinogens that are directly genotoxic leading to formation of chromosomal DNA-adducts. Both mechanisms could be involved in carcinogenesis [20].

Given that the eggs of *S. haematobium* secrete novel catechol-estrogens, and that in other contexts, estrogens are metabolized to active quinones that modify DNA [14], we now hypothesize that infertility during infection with *S. haematobium* might be related to the presence of parasite-derived catechol-estrogens. To begin to address this issue, which may have substantial implications for global health, here we undertook a cross-sectional study of residents of rural Angola, a region endemic for schistosomiasis haematobia [21]. A significant association between the presence of catechol-estrogens/ DNA adducts in the urine of females who were urine egg-positive for *S. haematobium* infection and self-reported infertility was detected.

Methods

Study area

Bengo province is a rural region, close to Luanda, the capital city of Angola. The study was performed in 2011 and 2012 at the Clínica da Sagrada Esperança, Luanda, Angola. This clinic serves the residents of Bengo. *S. haematobium* is endemic in this province; transmission occurs proximal to the Muxima River (Fig. 2). Bengo has a population of 500,000 inhabitants most of whom are involved with rural activities.

Study population and design

Irrespective of symptoms, 93 females willing to provide a urine sample were enrolled in the study. The ages of these participants ranged from 2 to 93 years. All participants provided oral informed consent. They all were advised that were free to withdraw from the study at any time, without the need for an explanation. Oral informed consent was documented by the physician performing the interviews. Written informed consent could not be obtained because participants were illiterate. Parents provided informed consent on behalf of participating children. Ethical approval for the study protocol, including the use of oral consent, was obtained from the review board (the local ethics committee) of Clinica da Sagrada Esperança, Luanda, Angola. The 93 volunteers were arbitrarily assigned to three age categories: 2–11 years (children), 37 participants; 12–19 years (adolescents), 17 participants; and 20–94 years (women), 39 participants.

Urine examination

Patients provided one sample of urine of ~50 ml volume, collected at the time of the interview. The entire micturition volume was filtered through a polycarbonate membrane of 14 μm mesh size and 25 mm diameter (Whatman plc, Springfield Mill, UK). Thereafter the membrane was stained with Trypan blue (Sigma-Aldrich Corp, St. Louis, MO). Schistosome ova retained on the membrane were identified with the aid of a light microscope, as described [22].

Medical history and clinical examination

Interviews were undertaken in a relaxed atmosphere. It was emphasized to the participants by the project leaders that the consultation was confidential and voluntary. Personal data were obtained in order to facilitate (subsequent) follow-up in the advent of subsequent appearance of cancer or other illnesses. Each participant was asked questions related to previous and present urinary system symptoms, gynecological complaints and general medical history and treatments. Regarding the fertility status, participants were assigned to one of three groups conforming to the WHO's definition of infertility: "Infertility is the inability of a woman to become pregnant after one year of sexual intercourse without using contraception, with primary infertility considered when the woman never conceived, and secondary infertility when she had had a previous labour" [23]. Thus, in accordance, groups were defined as those who reported absence of difficulties in becoming pregnant (Group 1); those who were unable to become pregnant after one year of trial (self-reported primary infertility - Group 2); and those who had previously borne one child but could

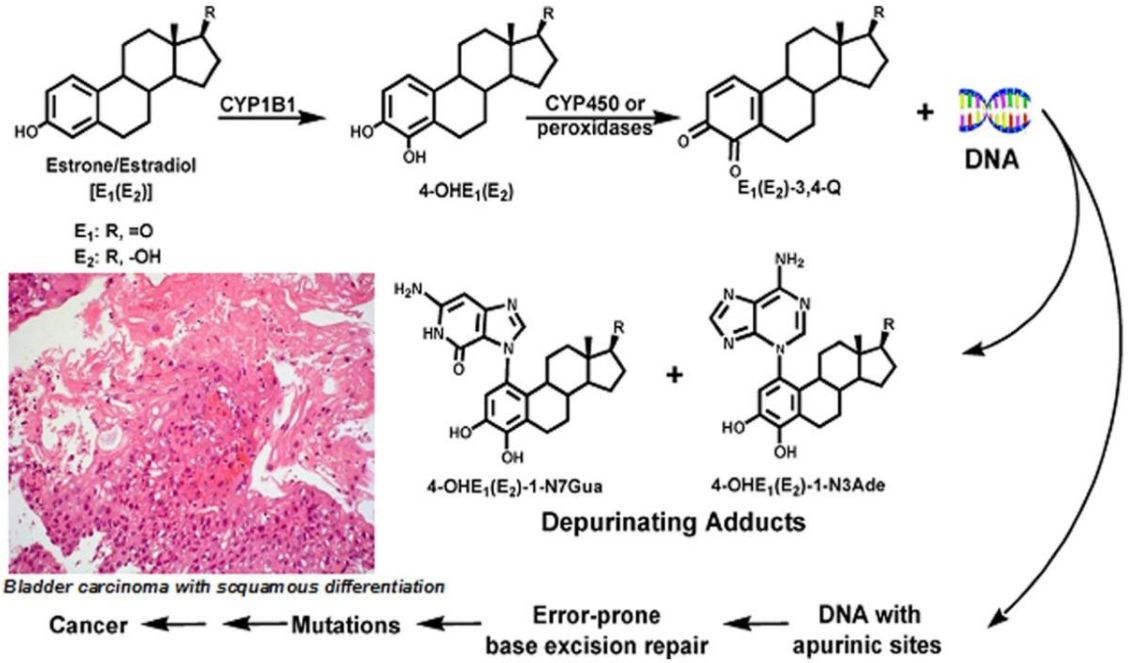

Figure 1. Major metabolic pathways in cancer initiation by estrogens. Adapted from [21,22].
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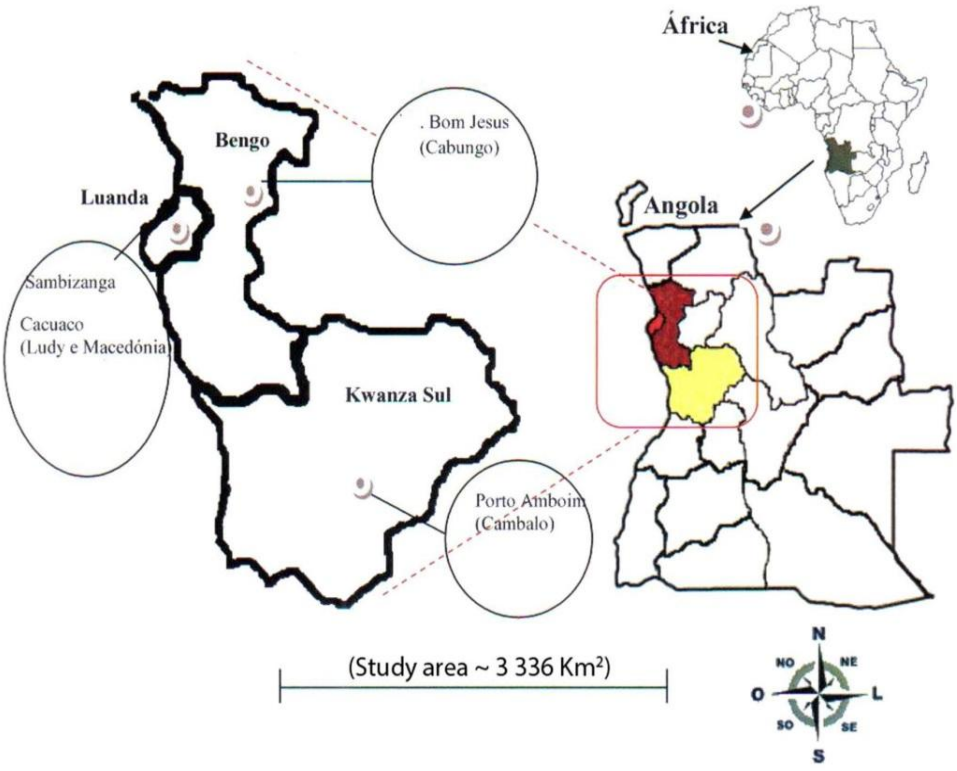

Figure 2. Map of Angolan study area. Adapted from [42].
doi:10.1371/journal.pone.0096774.g002

not achieve a second child (self-reported secondary infertility - Group 3). An urologist (JS) performed the interviews.

Liquid Chromatography Diode Array Detection Electron Spray Ionization Mass Spectrometry (LC/UV-DAD/ESI-MSn)

Analysis using LC/UV-DAD/ESI-MSn was performed on a Finnigan Surveyor Plus High-Performance Liquid Chromatography (HPLC) instrument (ThermoFinnigan, San Jose, CA) equipped with a diode-array detector and a mass detector. The HPLC system included a quaternary pump, autosampler, degasser, photodiode-array detector and automatic thermostatic column compartment, and was controlled by the Xcalibur software package (ThermoFinnigan). The mass detector was a Finnigan Surveyor LCQ XP MAX quadrupole ion trap mass spectrometer (ThermoFinnigan) equipped with an electron spray ionization (ESI) interface. Control and data acquisition were carried out with Xcalibur. Nitrogen gas of >99% purity was employed with a pressure of 520 kPa (75 psi). The instrument was operated in a negative-ion mode with an ESI needle voltage of 5.00 kV and capillary temperature of 325 °C. The full scan covered the mass range from 50–2,000 m/z (mass/charge number of ions). Mass spectrometry (MSn) data were simultaneously acquired for the selected precursor ion. Collision Induced Dissociation-Tandem Mass Spectrometry (CID-MS/MS) and MSn analyses were performed using helium with collision energy of 25–35 eV. The HPLC used a LiChroCART C18 column (125 mm×4 mm; 5 µm particle diameter, end-capped) with the temperature maintained at 25 °C; the mobile phase was composed of 1% (v/v) acetic acid in water (**A**) and acetonitrile, which had been degassed and filtered (**B**). The gradient used was 0–5 min with 100% **A**; 5–10 min, linear gradient from 100% to 80% **A**; 10–15 min, 80% **A**; 15–50 min, linear gradient from 80% to 40% **A**; 50–65 min, 40% **A**; 65–75 min, linear gradient from **A** to 100% **B**. The flow rate was 0.3 ml min^{-1} followed to split out in 200 µl min^{-1} to MS. Spectral data for all peaks were accumulated in the range of 200–600 nm. The instrument was calibrated with caffeine (Sigma-Aldrich), Met-Arg-Phe-Ala (MRFA) (tetrapeptide, Thermo Finnigan), and Ultramark 1621 (Lancaster Synthesis, Ward Hill, MA) in the mass range of 195–1,821 m/z. An advantage of inclusion of a MS detector over conventional HPLC analysis is that the number of channels in the detector can be set to specifically and separately identify all estrogen related compounds in a single injection of the urine sample.

Statistical analysis

Groups -- schistosomiasis positive and negative, presence or absence of metabolites -- were compared using the chi-square test with Yate's correction or with Fisher's exact, two-sided test when expected values were below 5. The chi-square test was used because it compares categorical variables. The data were normally distributed. Differences were considered statistically significant where $P \leq 0.05$ (VassarStats, Poughkeepsie, NY).

Results

Association between self-reported infertility and the presence of *Schistosoma haematobium* eggs in the urine

All 93 participants had lived in the area for at least two years. Urinary *S. haematobium* infection was diagnosed when schistosome ova were identified in the urine. The participants were assigned to one of two groups: positive and negative for *S. haematobium* infection. The median age was 22.9 (range 6 to 94) years for *S. haematobium*-positive and 27.3 (2 to 88) years for *S. haematobium*-negative females (Table 1). The fertility status of the women, excluding the prepubescent population (2–12 years old), and the presence of *S. haematobium* eggs in urine revealed 24 fertile and 29 women with a history of self-reported infertility --14 in Group 2, i.e. unable to become pregnant after one year trial, and 15 in Group 3, i.e. had borne fewer children than desired (Table 2). In general, signs and symptoms reported by the participants were distributed similarly between the groups, *S. haematobium* +ve and *S. haematobium* –ve, including dysuria, lower abdominal pain, history of water contact, etc. (Table 3).

Novel estrogen-like metabolites detected in urine of females positive for eggs of *S. haematobium*

Analysis of urine was undertaken using by LC/DAD/ESI-MSn. Inspection of the chromatography revealed several intense peaks, and four of these peaks corresponded to novel metabolites with catechol-estrogen-quinone radicals that, to our knowledge, have not been described previously. We analyzed the estrogen-related compounds using LC/DAD/ESI-MSn to yield the typical intensity spectra of the representative estrogen derivatives obtained by LC (Fig. 3). Each metabolite was detected and identified based on the parameters that were ostensibly unique to it, including the m/z value (m: atomic mass; z: atomic charge) and the retention time (RT). This produced the 'parent' mass spectrum by MS (parent m/z). Four new molecules were identified in *S. haematobium*-positive participants, at 28.35, 32.96, 43.47 and 44.22 RT (time at which a peak appeared). The corresponding RT of each peak was submitted to LC/DAD-ESI/MS and revealed a unique high peak in each -- 305.33, 269.33, 481.33 and 495.33 mz, respectively. These spectra were not observed in urine of non-schistosome infected participants (OR 3.35; 95% CI 2.32–4.84; $P \leq 0.01$). Thereafter, each of these peaks was submitted to ICD/MS/MS, which produced the mass spectra for the 'daughter' ions (daughters m/z) (Fig. S1). The collision at high pressure fragments the molecule at its most fragile sites, releasing several smaller molecules. Values of the RT, parent m/z, daughter m/z and the molecular mass of the four new catechols are provided in the Table S1. The chemical structure of each metabolite was reconstructed by overlaying the structures of the m/z's of daughter ions to ascertain whether they corresponded to the structure of each parent m/z (Fig. 4). In each case, full confirmation of the structural assignment for the four novel estrogen-like metabolites was established.

Estrogen metabolites of schistosomes in urine: an association with self-reported infertility

Comparisons between *S. haematobium* +ve and *S. haematobium* –ve participants revealed a significant association between presence of *S. haematobium* eggs in the urine and hematuria and presence of estrogen metabolites in urine and Group 2 (self-reported primary infertility) (Table 3). A significant association between self-reported infertility (Group 2+3) and *S. haematobium* estrogen metabolites in urine also was observed (Table 4).

Discussion

Female genital schistosomiasis (FGS) is common in rural regions of sub-Saharan Africa; of the estimated 70 million children currently infected with *S. haematobium*, approximately 19 million girls and women will develop FGS in the coming decade [24]. Up to 75% of girls and women with chronic *S. haematobium* infection may be affected by deposition of eggs with granulomas and sandy patches on the cervix and, moreover, histopathological interpre-

Table 1. Numbers of positive (*S. haematobium* +ve) and negative (*S. haematobium* -ve) urine samples for eggs of *Schistosoma haematobium* according to the age of the participants.

Age groups (years)	*S. haematobium* +ve	*S. haematobium* -ve	Total
median age (range)	22.9 (6–94)	27.3 (2–88)	25.1 (2–94)
2–11, i.e. children	18	19	37
12–19, i.e. adolescents	12	5	17
20–94	16	23	39
Total	46	47	93

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tation of biopsy specimens indicates that this increases the vascular density of the genital mucosa. [25]. The resulting FGS has been associated with contact bleeding, discharge, pain on intercourse, as well as diminished fertility [26,27,28]. FGS can also be a source of shame and stigma [26,29].

In this cross-sectional study of 93 girls and women, schistosomiasis haematobia was statistically associated with self-reported primary infertility in these participants residing at a rural site, Bengo province, Angola. The outcome corroborated findings from case reports of decreased fertility in women with schistosomiasis [26,27,28,29,30,31,32]. These reports had indicated the need for further investigation given they dealt with only two community-based studies [33,34]. Travelers who contract schistosomiasis haematobia in endemic regions may be also at risk of infertility [35,36]. FGS may induce pregnancy-related disorders, but the data available so far are insufficient from which to draw firm conclusions. Functional and anatomical disorders due to schistosome egg granuloma, including fibrosis of the ovaries and tubal obstruction, are established causes of infertility [37]. Hormonal disturbances during FGS may contribute to infertility and sub-optimal fecundity [38]. In this regard, it is relevant to note that *S. haematobium* expresses estrogenic molecules that down regulate the expression of estrogen receptors *in vitro* [11,13]

Manifestations of FGS in the young are difficult to evaluate because intra-vaginal inspection is only infrequently performed before the onset of puberty and/or sexual activity. Moreover, gynecological examination of people who have lived with these conditions for long periods requires appropriate cultural insight and skill in communication on the part of the investigators [26]. Accordingly, research in rural sites – including this present report -- is challenging, and requires the establishment of a differential diagnosis using non-invasive approaches [26]. Accordingly, development of non-invasive tests for FGS, especially given the potential for FGS- associated infertility, is a worthwhile goal that, if accomplished, can be expected to improve the public health in under-resourced and under-served populations. Based on the present findings, we predict that a diagnostic method based on mass spectrometric analysis of schistosome catechol estrogens in urine of *S. haematobium* infected persons could provide a novel, less invasive tool to complement current approaches.

Here, estrogen-like metabolites similar to those identified previously in adult worm and eggs stages of *S. haematobium*

Table 2. Status of fertility among Angolan women who provides urine samples positive (*S. haematobium* +ve) and negative (*S. haematobium* -ve) for eggs of *Schistosoma haematobium*, according to the age of the participants.

	N (%)	median age (years)	Age range (years)
Non-reproductive females	40 (100%)	8.25	2–12
S. haematobium +ve	21 (52.5%)	9.3	6–12
S. haematobium –ve	19 (47.5%)	7.1	2–11
Fertile women (Group1)	24 (100%)	51	18–94
S. haematobium +ve	8 (33.3%)	52.5	18–94
S. haematobium –ve	16 (66.7%)	50.25	19–88
Self-reported infertility (Group 2+3)[a]	29 (100%)	26.9	17–41
S. haematobium +ve	17 (58.6%)	25.6	18–41
S. haematobium –ve	12 (41.4%)	28.8	17–40
Self-reported primary infertility (Group 2)	14 (100%)	18.8	17–21
S. haematobium +ve	10 (71.4%)	19	18–21
S. haematobium -ve	4 (28.6%)	18.3	17–19
Self-reported secondary infertility (Group 3)	15 (100%)	34.5	24–41
S. haematobium +ve	7 (46.7%)	35.1	27–41
S. haematobium -ve	8 (53.3%)	34	24–40

[a]Women had been unable to become pregnant after one year of trial (self-reported primary infertility, Group 2) and those who had borne fewer children than they desired (self-reported secondary infertility, Group 3).

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Table 3. Symptoms and fertility state of 93 female residents of the Bengo region of Angola, an area endemic for schistosomiasis haematobia.

Symptom	*Sh egg* +ve; n = 46	*Sh egg* –ve; n = 47	OR	95% CI	*P*-value
Hematuria	35	22	3.9	1.6–9.8	≤0.01
Dysuria	26	23	1.43	0.63–3.25	0.27
Lower abdominal pain	24	22	1.3	0.57–2.95	0.34
History of water contact	37	39	1.1	0.38–3.14	0.54
Previous treatment with praziquantel	16	10	0.44	0.17–1.13	0.07
Estrogen metabolites	25	0	3.35	2.32–4.84	≤0.01
Fertility status					
Fertile	8/24	16/24	0.98	0.33–2.97	0.61
Group 2+3	17/29	12/29	1.02	0.34–3.07	0.60
Group 2	10/29	4/29	4.06	0.94–17.4	≤0.03
Group 3	7/29	8/29	0.4	0.10–1.40	0.12

Women unable to become pregnant after one year of trial (self-reported primary infertility - Group 2) and those who had borne fewer children than desired (self-reported secondary infertility - Group 3).
Sh egg +ve, positive for eggs of *S. haematobium* in urine; *Sh egg* –ve, negative for eggs of *S. haematobium* in urine.
OR, odds ratio; CI, confidence interval.
doi:10.1371/journal.pone.0096774.t003

[13,14] were detected by LC–MS in urine of study participants with FGS, and were statistically associated with self-reported infertility in the study volunteers. These electrophilic compounds can react with DNA to form the depurinating adducts. It is not inconceivable that apurinic sites in chromosomal DNA that result from this type of reaction generate mutations that might underlie infertility. Mutations in genes of the steroid enzyme pathway genes can lead to autosomal recessive infertility [39]. The aromatase enzyme, encoded by the CYP19 gene, converts the androgens testosterone and androstenedione to estradiol and oestrone, respectively. Therefore mutation in CYP19 gene results in estrogen deficiency in both females and males [39]. To date, we have not addressed male infertility caused by *S. haematobium* infection, but we aim to address this aspect in future studies in Bengo [6,40,41].

Even though our present report presents a statistical association between the presence of estrogen metabolites in the urine of *S. haematobium*-infected females and sub-fertility, several confounding variables may weaken our hypothesis. First, questions about gynecological complaints and sexual activities in adolescents were asked in the presence of parents; this may have led to a systemic underreporting of complaints and/or sexual activity in some of the participants. Second, primary and secondary infertility due to male-associated factors have not been analysed. Third, we did not have access to information about number of abortions and/or other associated gynecological diseases, or information concerning treatment for schistosomiasis received by the study participants. Given the context of the association between FGS and difficulty in becoming pregnant, the presence of putative mutagenic catechol-estrogens in *S. haematobium* infected persons may have practical implications for development of new approaches to control FGS and its sequelae. We have previously reported novel estrogenic molecules in *S. haematobium* and sera from cases of schistosomiasis haematobia [13,14]. We now present evidence, also by mass

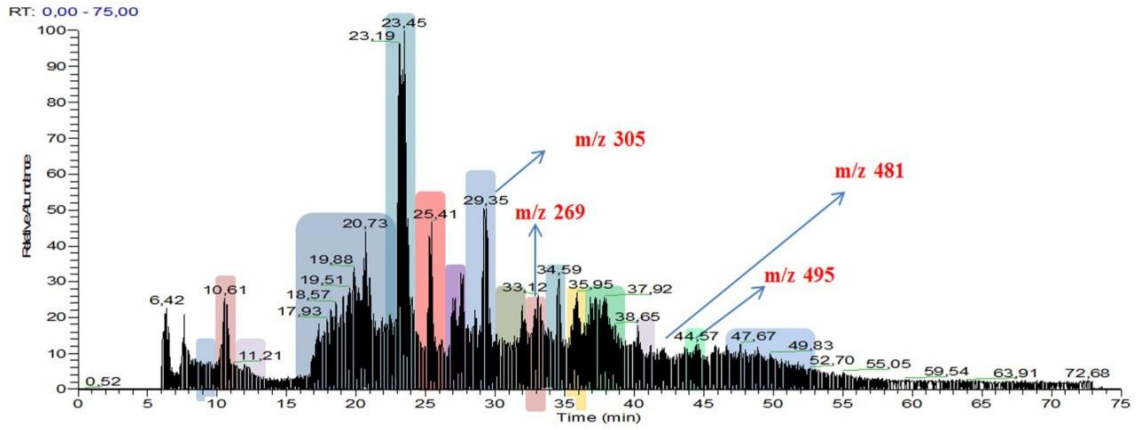

Figure 3. Typical spectra of representative estrogen metabolites, which were obtained in a single injection. The m/z of the molecules and the respective retention time at which they appear are highlighted.
doi:10.1371/journal.pone.0096774.g003

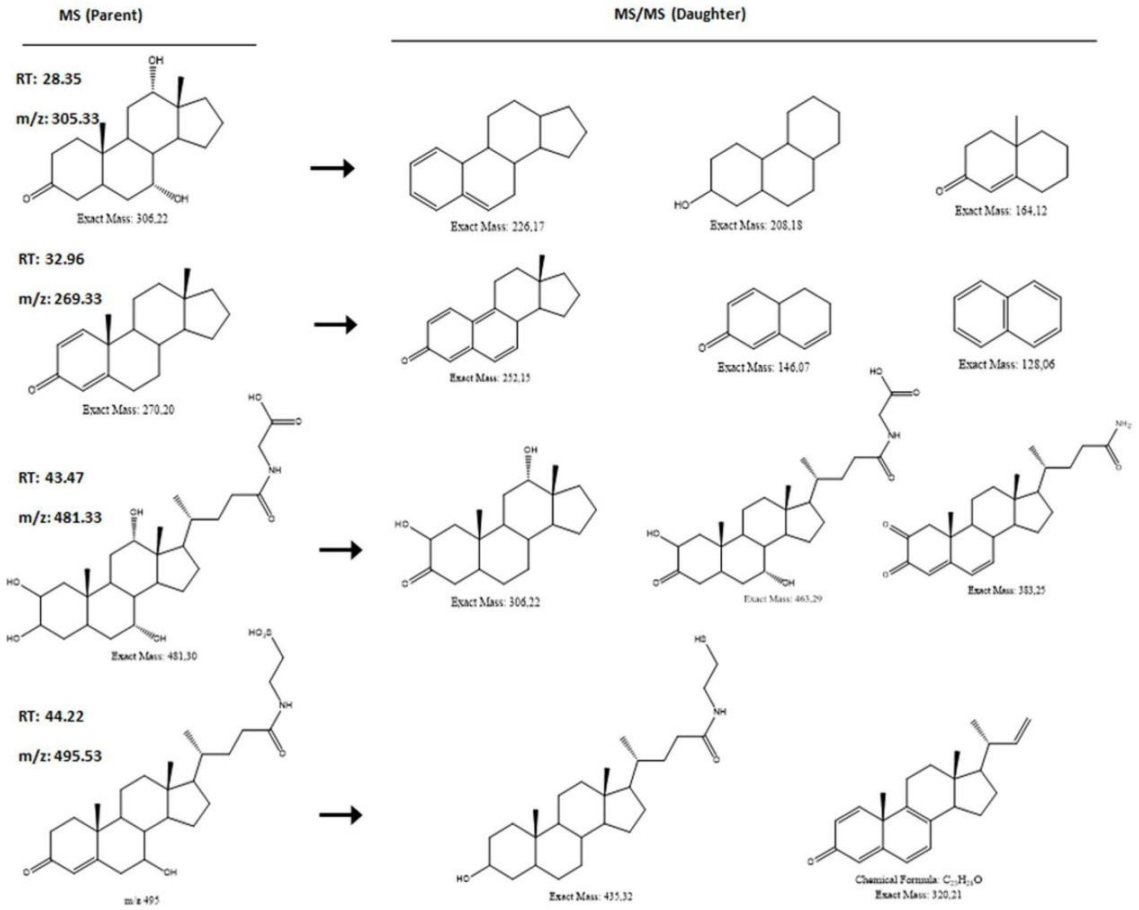

Figure 4. Chemical structures, retention times (RT) and m/z values for the four main components identified by LC–MS analysis of urine of *Schistosoma haematobium*-infected females. Atomic mass (m); atomic charge (z).
doi:10.1371/journal.pone.0096774.g004

Table 4. Numbers of positive (E+) and negative (E-) estrogen metabolites in urine samples according to the fertility status of the study participants.

	E + (n = 25)	E - (n = 21)	OR	95% CI	*P*-value
Fertile women (ages)	2 (29, 63)	6 (28–94)			
Group 2+3 (ages)	15 (19–41)	2 (21–34)	4.33	1.13–16.70	0.03
Group 2 (ages)	9 (18–20)	1 (21)	2.67	0.60–11.80	n.a.
Group 3 (ages)	6 (27–41)	1 (34)	4.75	0.51–44.50	n.a.
Total	17	8			
≤12 years	8	13			
Total	25	21			

Women unable to become pregnant after one year of trial (Self-reported primary infertility - Group 2) and those who had borne fewer children than desired (Self-reported secondary infertility - Group 3).
OR, odds ratio; CI, confidence interval.
doi:10.1371/journal.pone.0096774.t004

spectrometry, of the detection and characterization of similar molecules in the urine of *S. haematobium* infected people, of which the majority are catechol estrogen quinones.

The genotoxicity of estrogen metabolites might be attributed to oxidation of catechol-estrogens to quinones followed by redox cycling and formation of reactive oxygen species (ROS) that in turn react with DNA [42,43]. The metabolism of estrogens and the production of depurinating estrogen-DNA adducts as well as the generation of ROS can be implicated in a pathway underlying *S. haematobium*-promoted host cell DNA damage. The mutagenic effect of this estrogen-DNA adduct mediated pathway could explain the statistical association between *S. haematobium* infection and reduced fertility. Given that the genome and transcriptomes of eggs, female and male adult worms of *S. haematobium* is available [44], studies utilizing RNA interference and/or other functional genomic tools to silence components of estrogen catabolism pathways e.g. schistosome estradiol 17beta-dehydrogenase should be informative [45]. To conclude, given that the four novel estrogen-like metabolites identified here in urine were associated with both FGS and with difficulty in becoming pregnant, deeper investigation in larger numbers of infected people and in other geographical regions endemic for *S. haematobium* infection is clearly worthwhile.

Supporting Information

Figure S1 Mass spectra of catechol estrogen quinones from *Schistosoma haematobium* infected individuals by MS (parent m/z) and MS/MS (daughters m/z). (**A**) m/z 305.33; (**B**) m/z 269.33; (**C**) m/z 481.33 and (**D**) m/z 495.53. The top image corresponds to the m/z of the four estrogen metabolites with catechol-quinone radicals (A–D) found at the retention times (RT) illustrated in Fig. 3 and obtained by LC/DAD-ESI/MS. The maximum peak of each (arrows) was submitted to CID/MS/MS, where the collisions released the mass spectra of the "daughters" (bottom image: encircled). Atomic mass (m); atomic charge (z).
(PDF)

Table S1 Estrogen metabolites with spectrometric masses of parent and daughter ions and retention times.
(DOCX)

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Author Contributions

Conceived and designed the experiments: MCB. Performed the experiments: MJG NV MdLD. Analyzed the data: MCB MS GR PJB. Contributed reagents/materials/analysis tools: JS LLS CL AG JMTdS CO PX AB JMCdC PG. Wrote the paper: MCB MS GR PJB. Performed the interviews: JS LLS CL.

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ANEXO 3

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Tumour-like phenotypes in urothelial cells after exposure to antigens from eggs of *Schistosoma haematobium*: An oestrogen–DNA adducts mediated pathway?

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ABSTRACT

Chronic infection with the blood fluke, *Schistosoma haematobium*, is associated with squamous cell carcinoma of the bladder. Previously, it has been shown that soluble extracts of mixed sex adult *S. haematobium* worms (SWAP) are tumourigenic, both in vitro and in vivo. In addition, oestrogen-related molecules in SWAP of *S. haematobium* down-regulate oestrogen receptors (ERs) alpha and beta in oestrogen responsive cells. Moreover, schistosome oestrogens occur in sera of persons with schistosomiasis haematobia and repress transcription of ERs in urothelial cells. Given that eggs of *S. haematobium* are the developmental stage directly responsible for urogenital disease during schistosomiasis haematobia, we suspected that soluble antigens from *S. haematobium* eggs exhibit similar or more potent tumorigenic capacity. Here we investigated the tumorigenic potential of soluble egg antigens (Sh-SEA) of *S. haematobium* and the endocrine system in favouring parasitism by schistosomes. The findings confirmed that 6.25 μg/ml of Sh-SEA was enough to stimulate cell proliferation, reduce apoptosis and increase oxidative stress of Sh-SEA-exposed urothelial cells. In addition, genotoxic effects of Sh-SEA on these cells were determined by using alkaline single-cell gel electrophoresis (Comet). Furthermore, Liquid Chromatography Diode Array Detection Electron Spray Ionisation Mass Spectrometry indicated the presence of catechol-oestrogens in *S. haematobium* SEA. A prospective oestrogen–DNA adduct mediated pathway in *S. haematobium* egg induced bladder cancer is also discussed.

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1. Introduction

Schistosomiasis is a neglected tropical disease caused by blood flukes of the genus *Schistosoma*. The parasite is transmitted to humans from freshwater snails. Schistosomiasis is one of the major neglected tropical diseases and it is considered the most important of the helminth diseases of humanity in terms of morbidity and mortality. More than 200 million people in 76 countries are infected by schistosomes and 600 million others are at risk of infection. One hundred and twenty million people are considered symptomatic and 20 million have severe disease. The disease may be responsible for an half million deaths per year. No vaccines are available and treatment relies on a single drug, praziquantel (King et al., 2005; Gryseels et al., 2006; Hotez et al., 2008).

Infection is frequently asymptomatic and diagnosis might not be made until a long time after exposure. Adult worms dwell in blood vessels and release eggs that become embedded in the bladder wall, where chronic inflammation, granuloma formation and eventually squamous cell carcinoma (SCC) may be induced. Therefore, *Schistosoma haematobium*, with the liver flukes, *Opisthorchis viverrini* and *Clonorchis sinensis*, is classified as a Group 1 carcinogen by the World Health Organization's (WHO's) International Agency for Research on Cancer (Bouvard et al., 2009), although the cellular and/or molecular mechanisms linking fluke infections with cancer formation have yet to be defined (Sripa et al., 2012).

Bladder cancer is one of the more dire complications of chronic schistosomiasis haematobia (Parkin, 2006; Bouvard et al., 2009; Rollinson, 2009; King, 2010; Kjetland et al., 2012).

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Case report studies indicate that patients with schistosomiasis may develop bladder cancer earlier than uninfected people. The severity and frequency of the sequelae of urinary schistosomiasis and of its complications (urothelial cancers) depend on the intensity of infection (worm burden and tissue egg burden) and the duration of infection (Hodder et al., 2000; Herrera et al., 2005). A *S. haematobium*-associated bladder cancer incidence of 3–4 cases per 100,000 has been estimated (Shiff et al., 2006). Felix et al. (2008) showed that the occurrence of transitional cell carcinoma (TCC) of the bladder has supplanted SCC in Egypt following a major decline in the prevalence of urinary schistosomiasis. Such a decline in the pattern of this infection suggests the importance of schistosome-associated bladder cancer that, elsewhere, may be more widespread than is presently thought (Shiff et al., 2010). In recent progress on the understanding of the host–parasite relationship of schistosomiasis haematobia, a draft genome sequence for *S. haematobium* was reported (Young et al., 2012) and a mouse model of *S. haematobium* egg induced immuno-pathogenesis and fibrosis typically found in human urogenital schistosomiasis was described (Fu et al., 2012). It was recently reported that this schistosome is amenable to being cultured in vitro and transformed with nucleic acid probes. Additionally, the presence of an intact and active RNA interference pathway in *S. haematobium* was demonstrated (Rinaldi et al., 2011).

Previously our group reported that soluble extracts of adult *S. haematobium* worms induce tumourigenesis (Botelho et al., 2009b,c). Chinese Hamster Ovary cells (CHO) exposed to whole *S. haematobium* antigens (Sh) induced high cellular proliferation and sarcomas after skin inoculation into nude mice (Botelho et al., 2009c). In addition, Sh-treated CHO cells showed an increased S phase, decreased apoptosis, down-regulation of the tumour suppressor, p27, and upregulation of the anti-apoptotic protein, Bcl-2 (Botelho et al., 2009b). Recent findings also indicate that *S. haematobium* induces the malignisation of the urothelium in CD1 mice (Botelho et al., 2011). However, the cellular and molecular mechanisms implicated have not been fully described. Also, potential parasite carcinogenic components have been investigated by our group (Botelho et al., 2009a, 2010). Four estrogenic molecules have been described in the parasite and in the sera from infected patients. Our results are consistent with the existence of an estrogenic molecule that antagonises the activity of estradiol. We found evidence for this molecule as we identified and characterised, by Liquid Chromatography–Mass Spectrometry (LC–ESI-MS), new estrogenic molecules previously unknown, which were present in the extract of *S. haematobium* worms and sera from schistosome-infected patients.

Here we observed similar molecules in soluble egg antigens (Sh-SEAs) from eggs of *S. haematobium*. These oestrogen-like hormones are known as catechol-oestrogens (Cavalieri and Rogan, 2011). Metabolites of catechol-oestrogens lead to the formation of oestrogen–DNA adducts and genotoxity. Thereafter, loss of DNA adducts can lead to mutations that initiate cancer. Based on the findings presented here, we propose that oestrogen–DNA adducts pathways may underlie the association between *S. haematobium* infection and bladder cancer.

2. Material and methods

2.1. Animals

Eight-week-old female golden hamsters (LVG/SYR) and CD-1 mice were provided by Charles River (Barcelona, Spain). Animals were allowed to acclimate for 1 week under routine laboratory conditions before starting the experiments. They did not receive any treatment prior to the study. Hamsters were kept in separated cages and mice were kept in six-littermate cages. They were fed standard balanced food and water ad libitum. All of the animals were maintained at the National Institute of Health (Porto, Portugal) in rooms with controlled temperature (22 ± 2 °C) and humidity (55 ± 10%) and continuous air renovation. Animals were housed under a 12-h light/12-h dark cycle (from 08:00 h to 20:00 h). All animal experiments were performed in accordance with the National (DL 129/92; DL 197/96; P 1131/97) and European Convention for the Protection of Animals used for Experimental and Other Scientific Purposes and related European Legislation (OJ L 222, 24.8.1999).

2.2. Experimental infections

Urine samples were collected from *S. haematobium*-infected individuals. The individuals were living in Angola, an endemic area for schistosomiasis. Following instruction in midstream urine collection, urine samples were then collected from each individual. Informed consent from patients was obtained. *Schistosoma haematobium* infection was detected by microscopic observation of the eggs in the sediment of centrifuged urine. The eggs were hatched and with the resulting miracidia, snails from a susceptible species, *Bulinus truncatus* strain from Egypt (maintained in our laboratory), were infected. Cercariae were obtained from these snails (Gaubert et al., 1999). Golden hamsters and BALB/c mice were experimentally infected with 100 cercariae; control animals consisted of littermates.

2.3. Eggs of S. haematobium and preparation of Sh-SEAs

Schistosoma haematobium eggs and adults from an Egyptian strain maintained in the laboratory (Lewis et al., 2008) were obtained from the intestines and livers of infected hamsters, as described (Rinaldi et al., 2011) and stored at −80 °C. Subsequently, eggs were thawed to 4 °C in PBS and lysed by sonication. A soluble extract was obtained by ultracentrifugation of the sonicated eggs. An extract of adult worms was prepared as described (Botelho et al., 2010). The protein concentration of the supernatant was determined using a micro BCA protein assay reagent kit (Viana da Costa et al., 1998), and the supernatant employed as Sh-SEA of *S haematobium*.

2.4. Cell lines

HCV29 (normal urothelial) cells were cultured and maintained at 37 °C in a 5% CO_2 humidified atmosphere in RPMI medium (Sigma–Aldrich, Saint Louis, MO, USA) with 10% FBS and 1% penicillin/streptomycin (Sigma–Aldrich). Cells were passaged every 5 days. Cells were serum-starved overnight before treatments (Botelho et al., 2012).

2.5. Proliferation assay

The CellTiter 96 AQ non-radioactive cell-proliferation assay (Promega, Madison, WI, USA) was used to assess cell viability. The assay employs MTS (3-[4,5,dimethylthiazol-2-yl]-5-[3-carboxymethoxy-phenyl]-2-[4- sulfophenyl]-2H-tetrazolium, inner salt), a tetrazolium compound and the electron coupling reagent, phenazine methosulfate. Viable cells reduce MTS to formazan, which is detected at 490 nm using a spectrophotometer; formazan production is time-dependent and directly proportional to the number of viable cells. HCV29 cells were cultured in 0.1 ml of RPMI media in 96-well flat-bottomed plates. Cultures were seeded at 1×10^4 cells/well and allowed to attach overnight. After the indicated time of incubation with the appropriate medium, 20 μl of assay reagent were added per well, and cells were incubated for 1 h before measuring absorbance at 490 nm. Background absorbance

from the control wells was subtracted. Studies were performed in triplicate for each experimental condition (Botelho et al., 2009b).

2.6. Apoptosis

A terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labelling (TUNEL) assay was performed using the in situ cell death detection kit (Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. Nuclei were counterstained with DAPI (Roche Diagnostics). The percentage of TUNEL-stained nuclei was evaluated in relation to every DAPI-stained nucleus observed. Immunofluorescence was visualised under a fluorescence microscope (Olympus, BH-2, UK). The percentage of stained cells was evaluated by counting the cells stained with TUNEL divided by the total number of nuclei stained with DAPI at a magnification 200× field. One thousand nuclei were evaluated. Three independent experiments were performed (Botelho et al., 2009b).

2.7. Oxidative stress assay

Oxidative stress was analysed by evaluation of total (GSHt), reduced (GSH) and oxidised (GSSG) glutathione levels. The intracellular levels of GSH and GSSG in Sh-SEA-treated HCV29 cells were evaluated by the DTNB-GSSG reductase recycling assay, as previously described (Costa et al., 2007). After exposure to Sh-SEA, cells were lysed and proteins were precipitated with 5% $HClO_4$. Following centrifugation (16,000*g*, 10 min, 48 °C), the supernatant obtained was used for the determination of GSHt, GSH and GSSG by spectrophotometry at 412 nm.

2.8. Comet assay

After treatment, cells were washed twice with chilled PBS (Mg^{2+} and Ca^{2+}-free), centrifuged at 78*g* for 5 min and resuspended in PBS. Cell viability was >85% for the tested dose in this study as assessed by using Trypan blue dye-exclusion. The alkaline version of the Comet assay was performed as described by Singh et al. (1988) with minor modifications. Briefly, cells collected by centrifugation (78*g*, 3 min) and suspended in 60 μL of 0.6% low-melting-point agarose (LMA) in PBS (pH 7.4) were dropped onto a frosted slide precoated with a thin layer of 1% normal melting point agarose. Slides were placed on ice for 4 min to solidify the agarose. Coverslips were removed and slides were immersed in lysis buffer (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM TrisBase, 0.25 M NaOH, pH 10) for 1 h at 4 °C in the dark. After lysis, slides were placed on a horizontal electrophoresis tank in an ice bath. The tank was filled with 1 mM Na_2EDTA, 300 mM NaOH, pH 13 (electrophoresis buffer) to cover the slides. The slides were incubated for 20 min in the dark to facilitate DNA unwinding and alkali-labile site expression.

Electrophoresis was carried out for 20 min at 30 V, 300 mA (1.2 V/cm). The slides were then washed for 10 min with 1 ml of 0.4 M TrisBase, pH 7.5 (neutralising solution). Subsequently, gels were stained with 100 μl of ethidium bromide (20 μg/ml) and covered with coverslips for 20 min. After staining, slides were washed twice with ice-cold, twice distilled water for 20 min.

Slides were coded and examined by a 'blind' scorer using a magnification of 400×. One hundred randomly selected cells (50 per replicate) were examined for each dose. Image capture and analysis were performed with Comet Assay IV software (Perceptive Instruments, Bury St Edmunds, UK); percentage of tail DNA (%T) was the DNA damage parameter evaluated (Kumaravel et al., 2009). The percentage of DNA in the tail is the fraction of DNA in the tail divided by the amount of DNA in the cell multiplied by 100.

2.9. Liquid Chromatography Diode Array Detection Electron Spray Ionisation Mass Spectrometry (LC/UV-DAD/ESI-MS^n) analyses

The LC/DAD/ESI-MS^n analysis was performed on a Finnigan Surveyor Plus HPLC instrument equipped with a diode-array detector and a mass detector. The HPLC system consisted of a quaternary pump, an autosampler, a degasser, a photodiode-array detector, an automatic thermostatic column compartment and a computer with Xcalibur® software. The mass detector was a Finnigan Surveyor LCQ XP MAX quadrupole ion trap mass spectrometer equipped with an electrospray ionisation (ESI) interface. Control and data acquisition were carried out with the Xcalibur® data system (ThermoFinnigan, San Jose, CA, USA). Nitrogen > 99% purity was used with gas pressure of 520 kPa (75 psi). The instrument was operated in negative-ion mode with ESI needle voltage, 5.00 kV; ESI capillary temperature, 325 °C. The full scan covered the mass range from m/z 50 to 2,000. MS^n data were simultaneously acquired for the selected precursor ion. CID-MS/MS and MS^n analyses were performed using helium as the collision gas with a collision energy of 25–35 eV.

The HPLC used a LiChroCART® C18 column (125 mm × 4 mm; 5 μm particle diameter, end-capped) with the temperature maintained at 25 °C; the mobile phase was composed of (A) 1% (v/v) acetic acid in water and (B) acetonitrile, which had been degassed and filtered. The gradient used was 0–5 min, 100% A; 5–10 min, linear gradient from 100% to 80% A; 10–15 min, 80% A; 15–50 min, linear gradient from 80% to 40% A; 50–65 min, 40% A; 65–75 min, linear gradient from A to 100% B. The flow rate was 0.3 ml min^{-1} and split out 200 μL min^{-1} to MS. Spectral data for all peaks were accumulated in the range 200–600 nm. The instrument was calibrated with caffeine (Aldrich, USA), MRFA (tetrapeptide, Thermo Finnigan, USA), and Ultramark 1621 (Lancaster Synthesis, USA) in the mass range of 195–1,821.

2.10. Statistical analysis

Data were expressed as mean ± S.D. A Student's t test was used to assess the statistical significance of differences; $P \leqslant 0.05$ was considered statistically significant.

3. Results

3.1. Schistosoma haematobium Sh-SEA increased the proliferation of urothelial cells in vitro

To begin investigating the effect of Sh-SEA on cell viability and proliferation, HCV29 cells were seeded on 96 well plates, starved overnight, treated with increasing concentrations of Sh-SEA for 24 h, cultivated for 24, 48 and 72 h, and proliferation assessed using a MTS assay (Fig. 1). The growth curve showed that cells incubated in 6.25 μg/ml of Sh-SEA for 24 h proliferated significantly faster and more than control (untreated) cells. We obtained the same results at 48 and 72 h (data not shown). Unexpectedly, higher concentrations of Sh-SEA did not increase cell proliferation and indeed, concentrations of Sh-SEA ⩾ 25 μg/ml caused a reduction in proliferation (Fig. 1).

This outcome suggested that increases in both proliferation and overall survival in HCV29 cells were consequences of exposure to Sh-SEA, at least at the lowest tested concentration of Sh-SEA. Accordingly, this concentration (6.25 μg/ml) was used in the following investigations.

3.2. Sh-SEA decreased apoptosis of urothelial cells

To analyse apoptosis, HCV29 cells were seeded on 96 well plates, starved overnight and exposed for 24 h to 6.25 μg/ml of

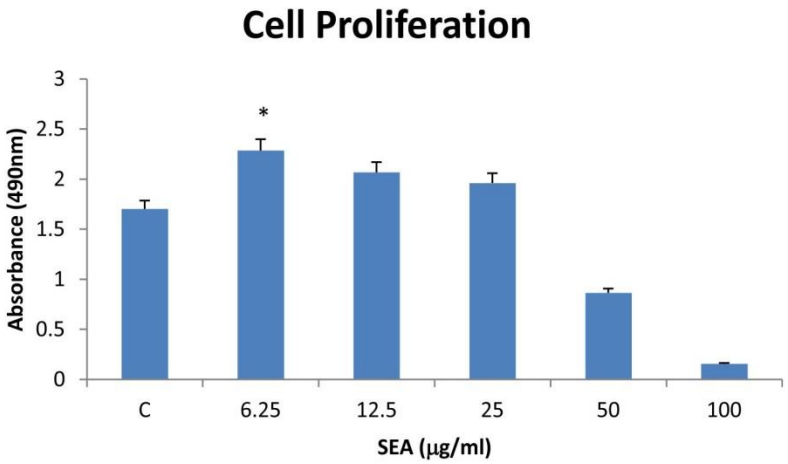

Fig. 1. Stimulation of human urothelial cells (HCV29) with soluble egg antigen (SEA) from *Schistosoma haematobium*. Cell proliferation in control (C) or SEA-exposed HCV29 cells at the indicated concentrations for 24 h, harvested 24 h later and analysed by Methosulfate Tetrazolium Salt (MTS) assay. Bars indicate the average of three experiments ± S.D. The asterisk indicates a significant difference ($P \leqslant 0.05$) compared with the control cells.

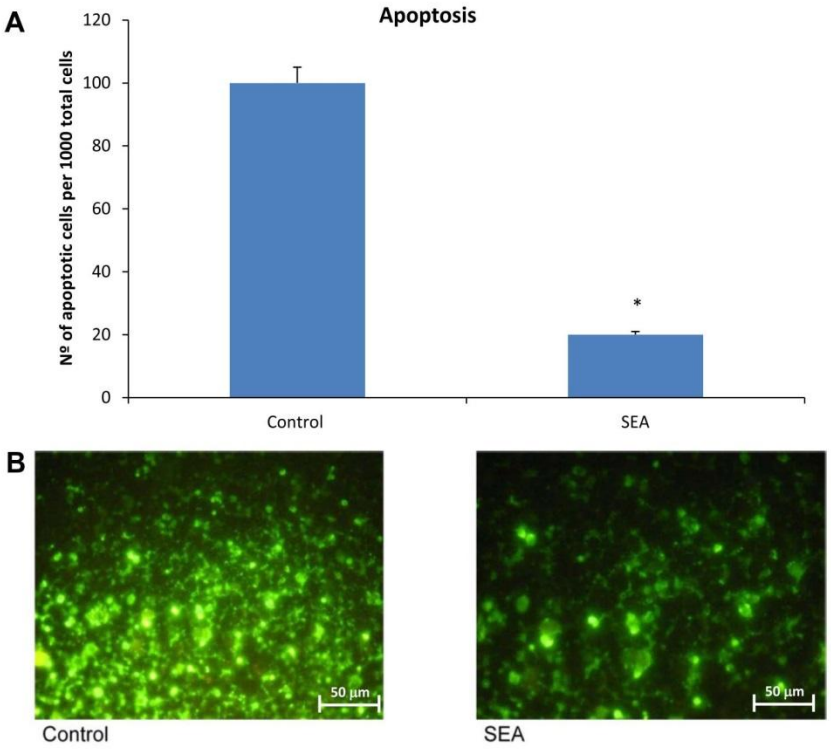

Fig. 2. Soluble egg antigen (SEA) from *Schistosoma haematobium* inhibited apoptosis in cultured human urothelial (HCV29) cells. (A) Apoptotic cells observed in control or SEA-exposed HCV29 cells at 6.25 µg/ml for 24 h, cultivated for 24 h and analysed by TUNEL. Bars represent the average of three experiments ± S.D. The asterisk indicates a significant difference ($P \leqslant 0.01$) compared with control cells. (B) Control and SEA-exposed HCV29 cells at 6.25 µg/ml as indicated. Scale bar = 50 µm.

Sh-SEA. Thereafter the cells were cultured for 24 h, harvested and processed for a TUNEL assay (Fig. 2). An increasing number of apoptotic cells per field in controls compared with cells exposed to 6.25 µg/ml of Sh-SEA for 24 h was observed (Fig. 2B). Cell counting resulted in a significant reduction in apoptosis in Sh-SEA-exposed cells compared with controls (Fig. 2A).

3.3. Sh-SEA increased oxidative stress of urothelial cells in vitro

Oxidative stress was determined by measuring oxidised glutathione (GSSG). A significant increase in GSSG levels was measured in Sh-SEA-exposed HCV29 cells compared with control cells. Fig. 3 shows the levels of GSSG detected in HCV29 cells after incubation

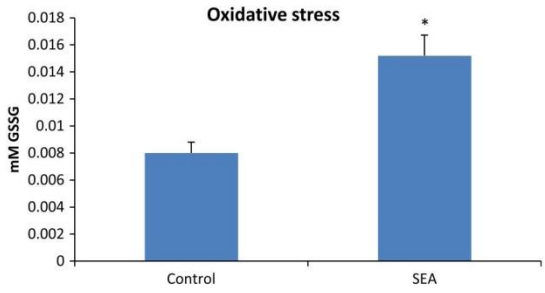

Fig. 3. Oxidative stress determined by measuring oxidised glutathione (GSSG). Oxidative stress levels measured in control or *Schistosoma haematobium* soluble egg antigen (SEA)-exposed HCV29 cells at 6.25 μg/ml of SEA. Bars represent the average of three experiments ± S.D. The asterisk indicates a significant difference ($P \leqslant 0.01$) compared with the control cells.

with 6.25 μg/ml of Sh-SEA and in control cells. These results showed that Sh-SEA treatment was able to elicit the alterations in glutathione status.

3.4. Induced genotoxicity of urothelial cells in vitro by Sh-SEA

Genotoxicity was detected by a Comet assay as described in Section 2.8. Fig. 4 shows an increase in tail intensity in Sh-SEA-treated cells compared with the control. Less damaged nuclei were observed in the control group of cells compared with cells exposed to 6.25 μg/ml of Sh-SEA (Fig. 4B). A 3.64-fold significant ($P < 0.05$) increase in the percentage of tail DNA was detected in Sh-SEA-exposed cells (47.34 ± 9%) compared with controls (25.19 ± 5%) (Fig. 4A).

3.5. Catechol-oestrogens are present in eggs of S. haematobium

HPLC with mass spectrometry was used to identify molecules in samples extracted from Sh-SEA. Fig. 5 depicts UV-chromatograms obtained for biological samples of *S. haematobium* mixed adults (Fig. 5A) and eggs (Fig. 5B). (Given we have previously employed LC–ESI-MS to analyse *S. haematobium* adult extracts (Botelho et al., 2010), extracts of adult worms were included here as a control (Fig. 5A).) In Fig. 6 we can observe the mass spectra (m/z) for the principal family of catechol-oestrogens.

4. Discussion

Previously we demonstrated that normal cells treated in vitro with *S. haematobium* total antigen display cancer-like phenotypes. Specifically, the cells present rapid uncontrolled division, high resistance to programmed cell death and an atypical capability to migrate (Botelho et al., 2009b) and, when injected into mice with no immune system, lead to the formation of tumours (Botelho et al., 2009c). We also demonstrated that *S. haematobium* total antigen in CD-1 mice normal bladders after intravesical administration of the parasite antigens induced inflammation and the development of urothelial dysplasia (Botelho et al., 2011). By contrast, here we characterised the effect of Sh-SEA in human urothelial cells (HCV29) using biological cell approaches typically

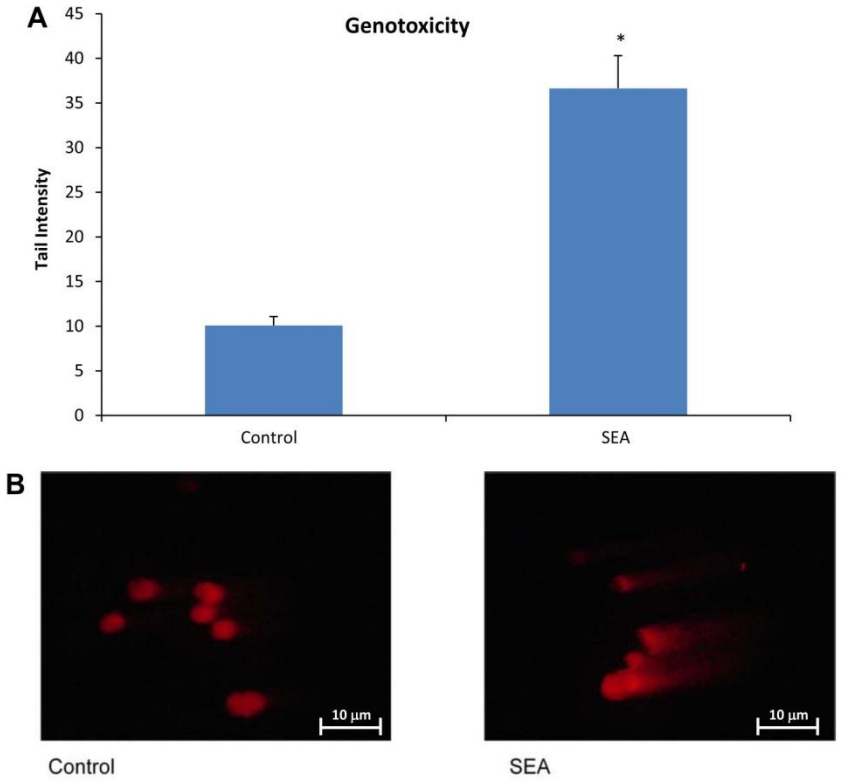

Fig. 4. Genotoxicity evaluated by a Comet assay. (A) Tail intensity detected in control and soluble egg antigen (SEA)-exposed HCV29 cells at 6.25 μg/ml of SEA. Bars represent the average of three experiments ± S.D. The asterisks indicates a significant difference ($P \leqslant 0.05$) compared with the control cells. (B) Less damaged nuclei were observed in control cells compared with SEA-exposed cells. Sacle bar = 10 μm.

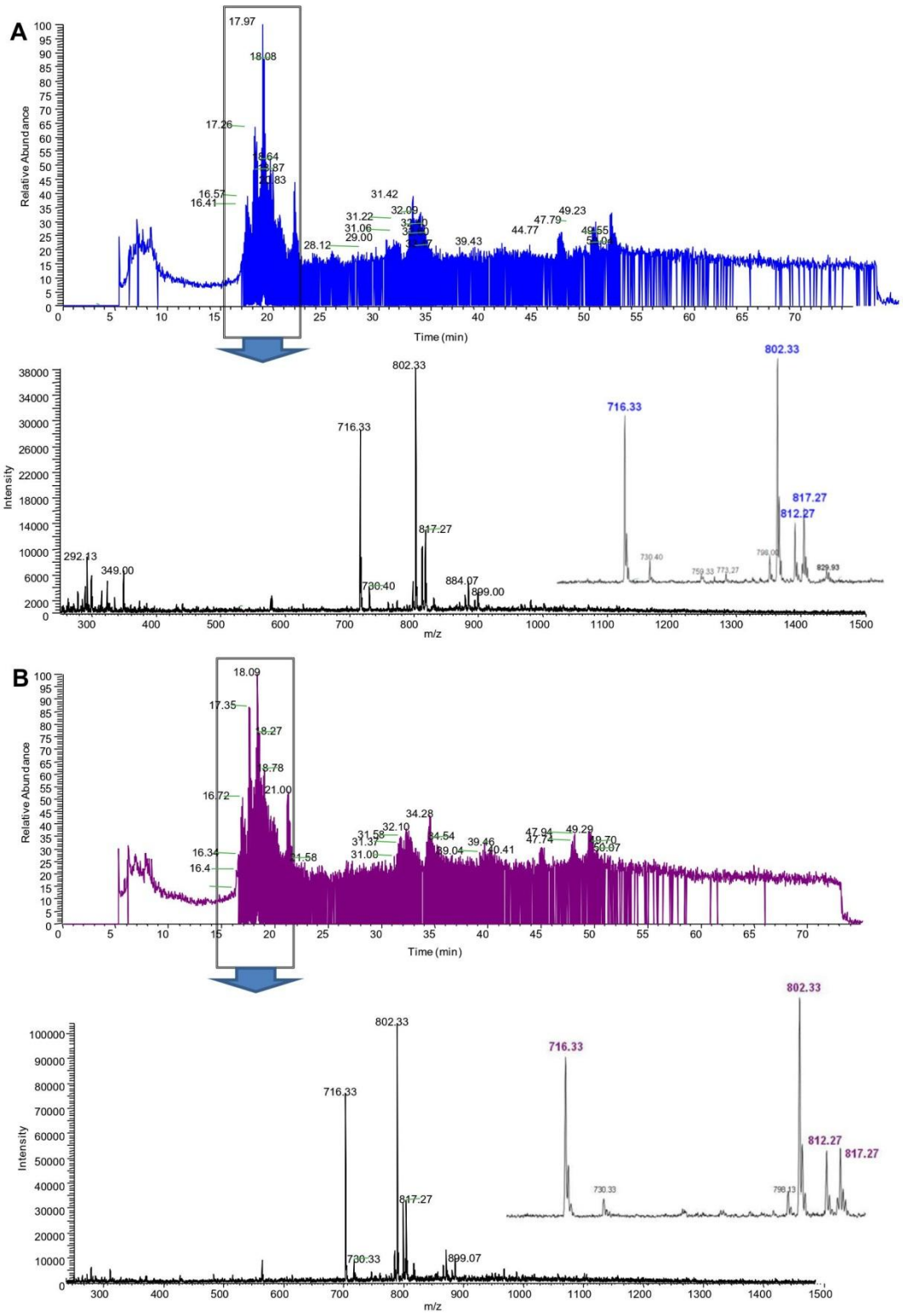

Fig. 5. HPLC with MS identifies molecules extracted from *Schistosoma haematobium* adult mixed sex worms (A) and *S. haematobium* eggs (B) as catechol oestrogens.

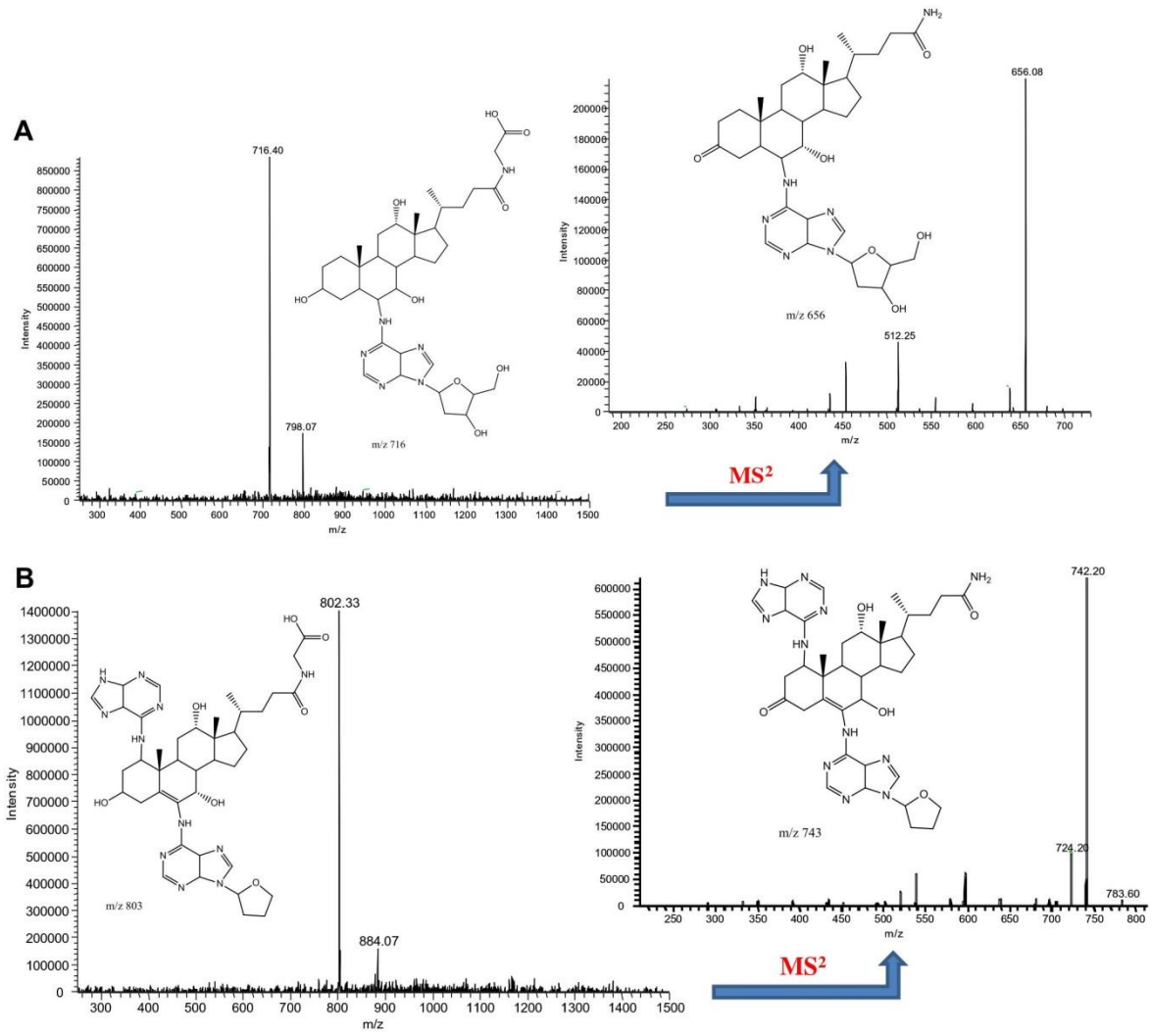

Fig. 6. Mass spectra of catechol oestrogens from *Schistosoma* haematobium and molecular structures of components by MS and MS/MS. The molecules are identified by their molecular weight (*m*/*z*). (A) *m*/*z* 716; (B) *m*/*z* 802; (C) *m*/*z* 812 and (D) *m*/*z* 817.

employed in studies of carcinogenesis. Hanahan and Weinberg (2000) presented the following in their findings, "The Hallmarks of Cancer": 'These are biological capabilities acquired during the multistep development of human tumors. The hallmarks constitute an organising principle for rationalising the complexities of neoplastic disease. They include sustaining increasing cell proliferation, apoptosis, inducing oxidative stress and genotoxicity'.

Cell cultures of HCV29 were exposed to Sh-SEA and showed that in a concentration range of 6.25–25 μg/ml, the extract increases proliferation. This observation is in agreement with findings with CHO cells treated with *S. haematobium* (Botelho et al., 2009a,b). It has been shown that prolonged stimulation of excessive proliferation of urinary bladder epithelial cells in rats leads to formation of carcinomas (Otori et al., 1997). However, higher concentrations of Sh-SEA did not stimulate cells to proliferate and indeed concentrations of Sh-SEA $\geqslant$ 25 μg/ml inhibited proliferation. Eggs of *Schistosoma mansoni* secrete a hepatotoxin. Cells infiltrate to surround the newly embolised egg, forming a peri-oval granuloma. In the T cell-deficient mouse, this granulomatous response is lacking, and toxic products released by eggs cause liver damage and death (Abdulla et al., 2011). Thus granulomata protect the host from toxic products of schistosome eggs. Despite the importance of this phenomenon in schistosomiasis mansoni, until recently (Fu et al., 2012), an informative model to analyse the issue in the context of *S. haematobium* SEA was not available. We aim to carry out studies in the future to address this issue.

Apoptotic cell loss in carcinogenesis has been examined by the TUNEL method (Takaba et al., 2000). We also used this method to analyse apoptosis in CHO cells after treatment with *S. haematobium,* where *S. haematobium* dramatically decreased apoptosis in CHO cells (Botelho et al., 2009b). Here, similar phenomena were seen in bladder epithelial cells exposed to SEA of *S. haematobium*.

Oxidative stress has been widely implicated as a mechanism underlying carcinogenesis. Numerous in vitro studies have identified increased reactive oxygen species (ROS) generation as an initiating factor in cancer. The generation of ROS and the resulting oxidative stress may cause a breakdown of membrane lipids, an imbalance of intracellular calcium homeostasis and DNA breakage

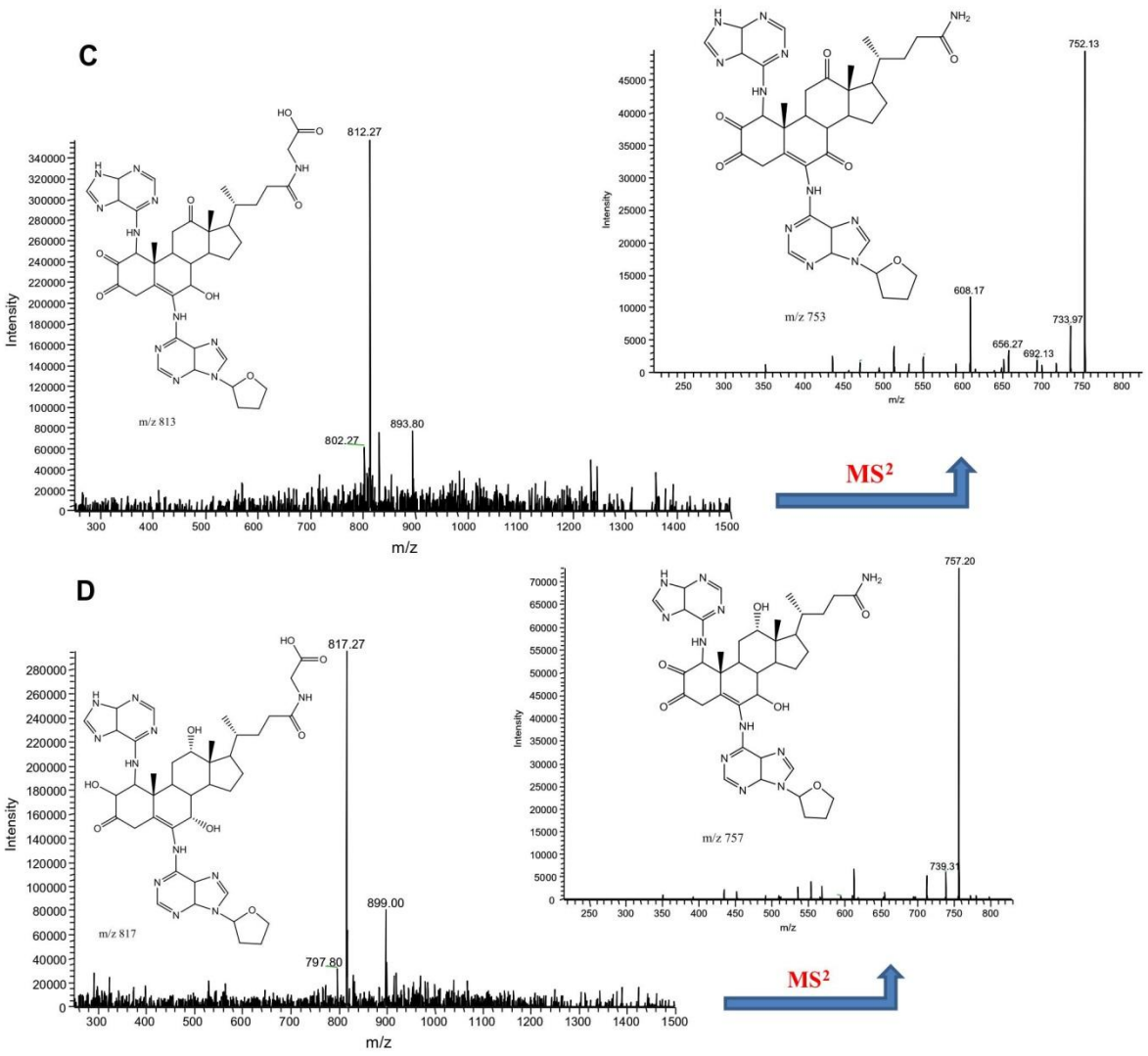

Fig. 6. (*continued*)

(Petruska et al., 1991; Clutton, 1997; Shukla et al., 2011). Here we report increased oxidative stress in Sh-SEA-treated cells. Earlier studies showed that *S. haematobium* infection is likely to cause bladder cancer by the same mechanism; Salim et al. (2008) suggested a strong correlation between *S. haematobium* infection and increased levels of oxidative stress accompanied by continuous DNA damage and repair in urothelial carcinomas. Biliary cell damage by *O. viverrini* likely stems from the actions of oxygen radicals such as nitric oxide (NO). NO not only induces DNA damage but has been reported to mediate DNA repair inhibition. Moreover, NO has also been demonstrated to inhibit apoptosis (Salim et al., 2008).

The DNA damage response is triggered by the detection of DNA lesions. This response consists of an orderly sequence of signal transduction events that can induce the accumulation of genetic errors which play a critical role in responding to various stresses that cause DNA damage, especially ROS (Matés and Sánche-Jiménez, 2000). We confirmed the genotoxic effects of Sh-SEA on bladder epithelial cells using alkaline single-cell gel electrophoresis (Comet). In the case of *O. viverrini* infection, DNA damage is caused in biliary epithelial cells while apoptotic mechanisms are deregulated, resulting in genetic alterations which may become fixed, leading to malignant transformation (Sripa et al., 2007). All of these manifestations facilitate carcinogenesis.

Studying the genotoxic molecular mechanism of Sh-SEA has helped elucidate pathways related to its tumourigenesis. The central hypothesis based on our studies is that genotoxic events and sustained signalling pathway stimulation drive deregulated cell proliferation and anchorage-independent growth; the processes are both required for mutations and progression towards neoplastic lesions, and play a role in Sh-SEA-induced mutagenesis and carcinogenicity. The well-known biological mechanisms, such as the alteration of cell-signalling pathways and induction of DNA damage, play a vital role in neoplasia induction (Huang et al., 2009). The initiation stage of carcinogenesis is mainly characterised by genotoxic processes, which may lead to irreversible changes in the structure of cellular genetic materials. Although DNA repair pathways exist for DNA restoration, however, erroneous repair and extensive DNA damage may cause mutations and ultimately

lead to cell transformation (Huang et al., 2009). Furthermore, since there is a link between DNA damage, mutations and cancer, Sh-SEAs that are potent in causing DNA damage can be regarded as more likely to have an effect on cancer development.

Given the context of the unarguable link between *S. haematobium* infection and bladder cancer, the presence of putative carcinogenic molecules in *S. haematobium* eggs identified here hopefully may have practical consequences for new approaches to control. We have previously identified, by MS in *S. haematobium* extracts and in the serum of infected individuals, four new estrogenic molecules that were formed by reactions of oestrogen-quinones with DNA (Botelho et al., 2010). In the present work we found evidence, as we identified and characterised by MS, similar molecules present in Sh-SEA. The majority of these compounds are catechol-oestrogens. Catechol-oestrogens are formed by hydroxylation on the steroid aromatic ring A. Hydroxylation of both C-2 and C-3 on a steroid ring was apparent and, further, oxidation into an estradiol-2,3-quinone. The genotoxic effects of oestrogen metabolites might be attributed to oxidation of catechol-oestrogens to quinones followed by redox cycling and formation of ROS that in turn react with DNA (Cavalieri et al., 1997; Lu et al., 2007).

To conclude, we anticipate that the findings will contribute to understanding how schistosomiasis haematobia leads to SCC of the bladder. Metabolism of oestrogens and the production of depurinating oestrogen–DNA adducts can be implicated in a pathway underlying *S. haematobium*-promoted host cell DNA damage. The carcinogenic effect of this oestrogen–DNA adduct mediated pathway could explain the link between *S. haematobium* infection and SCC of the bladder. Furthermore, LC/UV-DAD/ESI-MS^n emerges as an important tool to address eventual correlations between oestrogens and *S. haematobium*-associated bladder cancer. We recommend that future studies assess activities of specific catechol-oestrogens identified in schistosome eggs. We plan to follow this route using catechol-oestrogens purified from eggs of *S. haematobium* and/or synthetic versions of these putative carcinogens. In addition, studies utilising RNA interference to silence components of oestrogen catabolism pathways such as schistosome estradiol 17beta dehydrogenase and other catalysts should be informative (Rinaldi et al., 2011; Young et al., 2012)

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